FEAR, PAIN AND THE AMYGDALOID COMPLEX

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List of abbreviations

μA micro Ampere

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA Analyses of variance

AP-5 DL-2-Amino-5-phosphonopentanoic acid

BLA basolateral amygdala

CeA central amygdala

CNQX 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt

CS conditioned stimulus

dB decibel

FPS fear-potentiated startle

GABA γ-Aminobutyric acid

i.p. intraperitoneal injection

Isi inter-stimulus interval

L-NAME N^{ω}-nitro-L-arginine methyl ester

LTP long term potentiation

MR multi-receptive neurons

mV millivolt

NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NMDA *N*-methyl *D*-aspartate

PKA protein kinase A

PKC protein kinase C

SS shock sensitization

US unconditioned stimulus

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To my family and friends who over the years have so generously supported me.

ABSTRACT

In classical conditioning the amygdala is a critical area for the convergence of the unconditioned (US) and conditioned stimulus (CS). During this process the CS acquires some of the properties of the US. By assessing the US properties of foot-shock, namely reflex, pain and fear, the neural systems of pain and fear were evaluated in the rat basolateral and central amygdala. The central fear state produced by footshock was compared to the central fear state expressed during the fear-potentiated startle paradigm. By analysing the similarities and differences in the fear states, the effects of GABAergic, glutamatergic, and dopaminergic systems and protein synthesis inhibition on these fear states were investigated.

The basolateral amygdala was sensitive to GABAergic modulation during US and CS presentations. This was interpreted as a central fear effect. The central amygdala was sensitive to glutamate but not to GABAergic modulation. NMDA receptor antagonism prevented fear arousal to US but not CS presentation. This effect was interpreted as a deficit in pain processing. Non-NMDA receptor antagonism could significantly attenuate both US and CS fear expression. This was interpreted as an overall non-NMDA receptor inhibitory effect that affected pain and conditioned fear expression. Results of these experiments have implications for our understanding of the circuitry involved in processing the US. The basolateral amygdala appears to support emotional neural plasticity while the central amygdala appears to support pain neural plasticity. Finally and and most importantly each area processes different properties of the US.

Fear, pain and the amygdaloid complex

There is a fine line between useful and useless fear. What one person may perceive as exciting and fun may be perceived as dangerous and potentially harmful by others. One person may seek the heights of a mountain top while another may experience vertigo at climbing a small flight of stairs. Fear is expressed in many forms and intensities but it affects us all.

Fear is an adaptive feature that allows us to escape or confront predators and dangerous situations. When we hear an intruder our attention becomes focused on flee or fight, our reflexes become primed, pupils dilate and goosebumps occur so that we appear larger and fiercer. We may freeze, become mute and pretend we are dead or we can scream, startle and run away.

Thus the ability to experience and express fear is an adaptive feature that improves the chances of survival. Sometimes, however, fear expression becomes maladaptive, and this affects a significant portion of the population. In Australia about 2 % of the population suffer from fear-related conditions termed affective disorders (Andrews, 2006). Affective disorders are recognised and described in the Diagnostic and Statistical manual of Mental Disorders IV 2000 (American Psychiatric Association Task Force, 2000). These afflictions are all based upon fear, but have specialized features that distinguish one condition from another. The main distinguishing features are the fear provoking stimuli.

These can have multiple forms, be it real or imagined. For example, generalized anxiety disorder is usually based on unrealistic worry about general life experiences. Or social phobia has its fear based on interactions with people in public, obsessive compulsive disorder is based on internal fear based thoughts. Conversely, specific phobias have a more defined source such as spiders, and fear of heights. Thus, the fear source can have multiple forms but all can elicit fear responses. Fear can be defined as the emotional response to specific threats that may occur in future events. These events can include the expectation of pain and discomfort.

Not only do these disorders affect many people during their lifetime, 1 in 5, according to Andrews (2006), they also cost society in economic terms in the form of lost productivity and in on-going increases in medical costs (Andrews, 2006).

Some models of fear acquisition

The aforementioned disorders are all expressions of fear that at some stage have been learned, thus an association between threat and fear reaction has occurred. To understand fear acquisition, at least three models have been proposed. These are vicarious and verbal modelling and classical conditioning (Rachman, 1977). Two of the models do not involve a direct association between the threat and the fear response. The threatening information is supplied indirectly. In the first model the fearful behaviour is modelled by a conspecific and thus represents a form of vicarious learning. In the second model verbal information warns the recipient about potential danger. This information primes the receiver, who at a later stage is more likely to learn the association between the threat and the fear response. In the final model the association between the threat and the fear response is direct. For example, a predator is chasing prey, the prey escapes and has

learned to fear the presence of the predator. The formation of the association between predator and fear is a form of classical conditioning.

Fear acquisition – Classical conditioning.

This model has received considerable attention since Pavlov in 1904 (Pavlov, 1932) and Twitmyer in 1905 (Twitmyer, 1905) produced the concept more commonly known as classical or stimulus response conditioning. The main features of this model are that neutral and aversive stimuli are paired together in such a way that the order, timing and frequency of these pairings determine the strength of the association. The pairings occur in order; the neutral stimulus is presented first and followed closely by the aversive stimulus (Pavlov, 1932; Rescorla, 1968). If the order is reversed, a marginal association between the aversive and neutral stimuli occurs and the association is usually of an inhibitory nature (Pavlov, 1932; Rescorla, 1968). Similarly, if the time interval between exposure to the neutral stimulus and the aversive stimulus is long, conditioning is poor (Gallistel & Gibbon, 2000; Pavlov, 1932). The optimum timing is when the neutral stimulus is presented for a longer duration than the aversive stimulus, and co-terminates at the same time. Thus, an overlap between the neutral and the aversive stimulus exists. In addition, the frequency of these pairings is important; with more pairings the strength of the association increases (Gallistel & Gibbon, 2000). In addition, the evolutionary relevance of the neutral stimulus improves the rate of acquisition (Mineka & Ohman, 2002). Thus it is easier to acquire fear to large predators than it is to palatable food. And finally, the aversive stimulus needs to be sufficient but not excessive for conditioning to take place (Leaton & Borszcz, 1985).

During conditioning the first presentation of a neutral and usually novel stimulus does not elicit a particular response. Conversely, the presentation of the aversive stimulus produces a reliable and measurable internal physiological response. This physiological response is regarded as an internal or central fear state. Over time the novel stimulus becomes less novel with each paired presentation, and it comes to predict the onset of the aversive stimulus. Correspondingly, each aversive stimulus presentation elicits the central fear state. The critical point whereby learning has occurred is when the 'novel' stimulus can elicit the 'aversive' reaction without the aversive stimulus being present (Davis, 1986). In due course these pairings between stimuli produce neurological changes that can be measured.

A simple example often used in animal models of classical conditioning is the presentation of a conditioned stimulus (CS usually light or tone), and pairing this with an aversive stimulus, an unconditioned stimulus (US) which is typically a fear-producing foot-shock. Presenting the CS at a later date without the predicted US will still elicit the internal fear state (Davis, 1979, 1986; Pavlov, 1932).

Classical fear conditioning has considerable validity in the study of fear acquisition and expression, both in humans (Bitsios, Philpott, Langley, Bradshaw, & Szabadi, 1999; Grillon, 2002; Grillon & Baas, 2003; Grillon & Davis, 1997; Hijzen, Houtzager, Joordens, Olivier, & Slangen, 1995; Jovanovic et al., 2005), nonhuman primates (Winslow, Parr, & Davis, 2002) and in many other animals including rodents (Davis, 1986, 2006; Kurtz & Siegel, 1966).

Measures of fear

A problem with studying fear is that it is difficult to measure directly because it is an internal state. Animal models have assisted with understanding the changes in physiological and behavioural states associated with fear and these changes can be measured directly. Some of the physiological changes associated with fear are variations in respiration, heart-rate, blood pressure and analgesia (Helmstetter, 1992). These measurements always involve procedures that include handling the subject, which in itself can be aversive. Conversely, observing behavioural states can be more unobtrusive and may portray a more effective measure of the fear state. A plethora of research using animal models has quantified the behavioural responses associated with fear. A selection of these models is; conditioned suppression (Kim, Rivers, Bevins, & Ayers, 1996), ultrasonic vocalization (Brudzynski, 2001; Knutson, Burgdorf, & Panksepp, 2002), defensive burying (Pare, 1994; Treit, 1981), freezing behaviour (Fanselow, 1982; Fendt & Fanselow, 1999) and reflex potentiation (Davis, 1986, 1990; Greenwald, Bradley, Cuthbert, & Lang, 1998)

Conditioned suppression involves lever pressing for food, which is then interrupted by the presentation of a conditioned aversive stimulus. The duration of the interruption is a measure of fear (Kim, Rivers, Bevins, & Ayers, 1996). This paradigm involves the suppression of learned behaviour, which implies inputs of higher cognitive functioning, and is thus a relatively complex but effective method to study fear. Another method is the defensive burying behaviour, whereby rats bury a shock probe in bedding. The rate of burying is a measure of fear and anxiety (Pare, 1994; Treit, 1981) and involve more than one behaviour and the control of these require higher cognitive functioning. Simpler

methods are defensive freezing behaviour whereby the animal suppresses any ongoing behaviour and becomes immobile. Freezing behaviour can easily be detected for it involves lack of movement. But determining when mobility ceases or continues makes it problematic to accurately measure. Furthermore, pharmacological intervention cannot always distinguish between freezing and ataxia. While both involve immobility the cause for this can be very different (Davis, 1998). More importantly freezing behaviour is suggested to be the expression of a learned association which implies a memory component (Fanselow, 1982; Fendt & Fanselow, 1999). Although freezing behaviour is a valid measure, it is not the simplest.

A different natural behaviour that is indicative of fear expression is ultra sonic vocalization (Knutson, Burgdorf, & Panksepp, 2002). Rats readily engage in ultra sonic vocalization when confronted with predators, but also during laboratory settings when exposed to aversive stimuli, (Borszcz, 1993; Borszcz & Leaton, 2003; Lee, Choi, Brown, & Kim, 2001; Oliveira & Barros, 2006). This type of vocalization is said to be an expression of an unconditioned response (Illich, King, & Grau, 1995) and of pain (Oliveira & Barros, 2006).

Studying unconditioned responses instead of conditioned responses simplifies the paradigm and reduces the cognitive input a rat can contribute to fear expression. The final unconditioned measure of a central fear state to be considered is acoustic startle. The startle response is a reflex and not an emotion (Ekman, Friesen, & Simons, 1985). But, the reflex can be modified by an emotion (Kurtz & Siegel, 1966). For example, the increase between acoustic startle responses before and after presentation of an unconditioned stimulus, such as foot-shock, is a measure of fear (Boulis & Davis, 1989;

Davis, 1989). The fear produced by the aversive foot-shock enhances the startle reflex (Brown & Jacobs, 1949; Kurtz & Siegel, 1966). Furthermore, the acoustic startle response can be used to measure the expression of learned fear acquired during classical conditioning paradigms (Davis, 1986). The acoustic startle response is an effective means for establishing the level of fear because humans (Bradley, Lang, & Cuthbert, 1993; Grillon, 2002; Grillon & Baas, 2003; Grillon, Baas, Lissek, Smith, & Milstein, 2004; Jovanovic et al., 2005) and most animals studied in this respect exhibit startle (Davis, 1989; Landis & Hunt, 1939; Winslow, Parr, & Davis, 2002).

Conditioned suppression, defensive burying, ultrasonic vocalization, freezing and startle are all natural behaviours that become augmented during a central fear state. Furthermore, these behaviours can all be used to investigate various paradigms based on classical conditioning whereby the CS and US become associated and the CS presented at a later date will elicit components of the US. For the formation of the CS/US association the US must have specific properties (Borszcz, 1993).

US properties

During the last decades we have learned much about how we acquire fear. We have developed robust animal models which help to ascertain how we learn, express and 'overcome' fear. A challenge for classical fear conditioning is that when learning the relationship between the CS and the US, the US must have certain specific properties. The main property is that it must be aversive. Some typical examples used in rodent research are loud noise, tail-or foot-shock exposure. Secondly, it must be able to elicit physiological responses.

Analyses of tail and foot-shock reactions suggest it provokes stimulus-response elements such as spinal reflexes and pain (Illich, King, & Grau, 1995), but also an affective-motivational component (Borszcz, 1993, 1995). The measurements of some of these components present challenges. Spinal reflexes can be directly measured via the vigour of the motor-reflex. Pain can be measured via the emission of ultra sonic vocalization during shock (Illich, King, & Grau, 1995). But the affective-motivational component is difficult to measure directly.

Of the three components, it is the quality of the affective-motivational component that is proportional to the strength of the US-CS association (Borszcz, 1993; Leaton & Borszcz, 1985). Thus, increased fear creates stronger associations between CS and US. The quality of the spinal motor-reflex, i.e. the height of a jump/flinch elicited during shock, is not an indicator for the development of the strength between the CS and US association (Borszcz, 1993). Thus, the height jumped does not indicate the formation of fear associations. The amount of pain experienced may not necessarily be directly related to the strength of the US/CS association either. Moreover, it is difficult to show disassociations between pain and fear. Recent reports indicate that visceral and plantar pain can produce conditioned place aversion, however, the pain-fear connection could not be totally separated (Tanimoto, Nakagawa, Yamauchi, Minami, & Satoh, 2003). Similarly, pain produced by formalin injections resulted in ultrasonic vocalization at the 22 kHz range (Oliveira & Barros, 2006). This range relates to negative affect (Knutson, Burgdorf, & Panksepp, 2002). Whether pain without emotion can serve as an US is still unclear.

As noted earlier for an US to be conditionable it must have characteristics that support conditioning (Borszcz & Leaton, 2003). Of the three components produced by shock i.e. motor reflex, pain and emotion, it is the emotive element that supports conditioning. It is this element that is measurable after termination of the foot-shock. For example, vocalization after discharge is a component of the behaviours displayed once tail-shock has terminated. It is this component and not the reflex or the vocalization during tail-shock (pain) that is expressed during later fear tests. Similarly, fear-potentiated startle is an expression of the emotive element.

Borszcz and Leaton (2003) explained this phenomenon from a neuro-axial organizational point. This type of organization summarizes at which point in the central nervous system these functions occur, starting at the periphery and ending at a cortical level. Thus following the path of tail-shock in relation to vocalization after discharge, the sensation of shock starts at the periphery and travels to the spinal cord, where the jump/flinch reflex is processed at a spinal level and vocalization during tail-shock is mediated at the medullary level. But vocal after-discharge is mediated at a limbic level including the amygdala. This suggests a sequential model, in which different elements of tail-shock are processed at different neuraxial points. Analogous to this is foot-shock, of which the jump/flinch reflex is controlled at the spinal level (Boulis & Davis, 1989; Davis, Gendelman, Tischler, & Gendelman, 1982; Koch & Schnitzler, 1997), with pain distributed from the medulla (Gebhart, 2004) to the cortical and thalamic areas located before the amygdaloid complex (Shi & Davis, 1999), and the affective component being mediated in the temporal lobe including the amygdala (Meagher et al., 2001). There have been some attempts to locate the areas responsible for processing the motor-reflex, pain

and the affective-motivational responses during foot-shock, but little is known about the underlying neural systems associated with this.

Measuring the US effect using the shock sensitization paradigm.

A simple design that analyses the effect of foot-shock is the shock sensitization paradigm designed by Davis (1989). In a typical design rats are presented with 20 baseline white-noise bursts followed by 10 rapid foot-shock presentations which are then followed by 20 more white-noise bursts. Noise bursts produce startle and foot-shock produces stimulus-response and affective-motivational effects. The affective element increases the startle response. Thus, a significant difference between the baseline noise bursts and the post-shock noise bursts indicate a level of fear. This increase in fear can also be elicited in humans using shock and by exposure to unpleasant pictures (Greenwald, Bradley, Cuthbert, & Lang, 1998) indicating that this paradigm applies to various species. An extension of this paradigm is to precede the shock by a CS, such as tone or light, thereby representing the classical fear conditioning paradigm.

Measuring the CS effect using the fear-potentiated startle paradigm.

The fear-potentiated startle paradigm is a robust and validated measure to investigate fear to CS presentation (Davis, 1986; Kim & Jung, 2006). After several CS/US pairings the presentation of the CS elicits a central fear state (Pavlov, 1932). This central fear state can be measured during later testing whereby acoustic startle is presented before and after CS presentation. The difference between pre and post CS startle is accepted as a measure of fear (Davis, 1986; Kim & Jung, 2006).

Similarities between US and CS fear expression.

It is likely that the emotive element elicited by foot-shock and reflected in an increase in startle during the shock sensitization paradigm is the same as the emotive element elicited during the presentation of a CS. Both paradigms elicit a central fear state which may have the same underlying neural substrates. If so, then neural systems that affect the expression of fear during shock sensitization should also affect fear expression during fear-potentiated startle. However, not all neural systems that affect the expression of fear-potentiated startle will inhibit shock sensitization.

Differences between US and CS fear expression.

Of the three elements evoked by foot-shock, namely jump/flinch reflex, pain and emotion, the reflex and pain are unlikely to be elicited during CS presentation. No evidence of the jump/flinch reflex is shown during CS presentation (Davis 1998). Moreover, it is unlikely that the CS stimulates nociceptive receptors responsible for transmitting pain. Thus the only element produced by a US that is included during CS presentation is the emotive element.

During CS presentation additional elements are elicited that are not present during US presentation. Examples would be attention to and interpretation of the CS (Pezze & Feldon, 2004) and memory traces that may include the neural connections between CS and a central fear state (Stanton, 2000), and perhaps memory for the pain produced by foot-shock during the CS/US conditioning (Borszcz, 1995).

Previous work comparing central fear states.

The idea that there could be differences and similarities between two fear paradigms is not new. Rosen (2004) compared predator odour as the unconditioned stimulus and chamber context as the conditioned stimulus. His measure of fear was freezing duration. Simple paradigms were used. After adaptation to the test chambers, rats were either exposed to a single foot-shock thus pairing context and foot-shock (context = conditioned stimulus, CS, shock=unconditioned stimulus, US), or they were exposed to context and an artificial fox odour (context =CS trimethylthiazoline, TMT = US). Freezing behaviour was measured immediately after presentation of the stimulus. The rats showed significant freezing responses after presentation of foot-shock or TMT. But the TMT-exposed group failed to produce freezing behaviour 24 hours later when re-exposed to the context (Wallace & Rosen, 2000). Thus, no lasting association had formed between context and TMT exposure. Conversely, foot-shock exposure reliably produced conditioning, thus forming a context-foot-shock association which was expressed as freezing behaviour 24 hours later. Even though TMT produced immediate freezing it did not produce a long-term association between TMT and context, thus violating one of the fundamental principles of a US (Borszcz, 1993, 1995; Borszcz & Leaton, 2003). A further problem was the measure of freezing behaviour used by Wallace and Rosen (2000). Although freezing is a valid index of fear it is difficult to determine if a rat is expressing fear or some other unrelated state. Thus, comparing TMT exposure to contextual conditioning may not be robust enough for investigating the properties of an US and then comparing these to the properties of a CS.

Another investigation of the overlap between a US and CS was that of Walker and Davis (Walker & Davis, 1997a, 1997b). They explored whether or not light-enhanced startle reflected unconditioned fear, and compared this with fear-potentiated startle. The advantage of using startle as a measure of fear is that it is a reflex and thus difficult to suppress (Davis, Gendelman, Tischler, & Gendelman, 1982). Startle is elicited by noise presentation and the reflex is enhanced by fear arousal. During the light-enhanced startle paradigm rats were exposed to a brightly lit environment and showed significant augmented startle responses after noise probes. The second paradigm consisted of 20 CS (light)/US (footshock) trials. After conditioning the CS elicited significant increases in startle.

Both paradigms used the same visual stimulus to evoke fear and used a change in startle amplitude to measure this. But a significant difference was that in the light-enhanced paradigm the bright-light was the US, and this was used to provoke unconditioned fear. However, during the second paradigm the US was footshock. If bright-light exposure produced fear then it should also be suitable for an US. Therefore, ideally Walker and Davis (1997 a, b) should have exposed rats to a CS such as a tone and paired this with bright-light exposure, instead of foot-shock. If rats showed reliable fear conditioning to the tone at a later date then this would indicate that bright-light exposure is indeed a valid measure of unconditioned fear. A weakness in this research was that two types of US were used, bright-light (as US for testing unlearned fear) and foot-shock (as US for light-shock association), but using light to test for CS/US association.

In this thesis, it is suggested that a more accurate comparison may be drawn between unconditioned and conditioned fear by using a comparison between the shock

sensitization and the fear-potentiated startle paradigms. Because both paradigms use foot-shock to elicit fear and increased startle responses to measure this, extra variables that could potentially influence the results are kept to a minimum.

In summary, this thesis explores the differences and similarities between US- and CS-provoked fears. To understand the differences and similarities, particular brain regions were investigated, specifically the amygdala. A plethora of research has been conducted on this area, and has confirmed that the amygdala is the point of convergence for the CS/US association (Maren, 1999a; Maren, Yap, & Goosens, 2001; Romanski, Clugnet, Bordi, & LeDoux, 1993; Shi & Davis, 1999). Therefore, it was deemed important to evaluate the role of specific neural systems in US/CS-provoked fear by intracranial infusion of various types of neurotransmitters into the amygdala during fear expression to US or CS presentation. In the following section the most important amygdaloid nuclei and associated neural systems and their role in fear processing are discussed.

A general overview of the amygdaloid complex.

Klűver- Bucy (1938) discovered that damage to the limbic system and the temporal lobes reduced fear expression and altered behaviours such as sexuality, emotionality and produced poor maternal behaviour. Further analyses by many researchers showed that damage to the amygdala was the cause of this. The amygdaloid complex is not only involved in processing fear related behaviours but also in other emotionally connected functions. For example, the amygdala has been implicated in social cognition in humans and primates (Adolphs, 1999). Social cognition has an important function in groups with

complicated social structures. The ability to judge the emotional state of others makes for smoother and less provoking social interactions.

The amygdala is a structure that is incomplete at birth and develops further during maturation. However, deficits in early post-natal amygdalar development may be an underlying cause in autism (Bachevalier & Loveland, 2006). Autism is characterised by impaired play behaviours, social interactions, verbal and non-verbal incompetence, stereotypical and repetitive behaviours (Schultz, 2005). Towards the end of our lifespan the amygdala plays an important role in Alzheimer's disease and other dementias. For example, accelerated volumetric deterioration of the amygdala can be seen in early stages of Alzheimer's disease (Mizuno, Wakai, Takeda, & Sobue, 2000). A direct relationship was found between volumetric amygdalar deterioration and impairment for recalling memorable events (Mori et al., 1999). This indicates a complicated role for the amygdaloid structures during various episodes in our lifetime.

More importantly, the amygdala is notable for its role in both negative and positive emotions. These emotions can be linked to daily activities that promote survival, such as remembering pleasantly edible foods, but also remembering the disgustingly inedible ones. The amygdala, for example, is involved in drug addiction (Koob, 2003) but also taste aversion (Yamamoto, Fujimoto, Shimura, & Sakai, 1995). Drug addiction involves associations between drugs and pleasurable feelings and this link is initially made through activity of the amygdala (Koob, 2003). Over time drug use may become a habit that is controlled by the higher functioning frontal cortex, and after this practice is turned into an addiction it is once again governed by the amygdala (Everitt and Robbins, 2006). Taste aversion involves associative learning between sustenance and visceral distress and

thus unpleasant feelings (Yamamoto, Fujimoto, Shimura, & Sakai, 1995). A final example of amygdaloid functioning is that it is involved in negative emotional learning and expression of these emotions in animals and humans (Cheng, Knight, Smith, & Helmstetter, 2006) of which the most studied major negative emotion is fear. In summary, the action of the amygdaloid complex is not only diverse but also obligatory for successful functioning and survival.

Neuroanatomy of the amygdalar divisions

In this section the two major amygdaloid structures studied in this thesis are described; these are the rodent central and basolateral amygdala. Their general role in the generation of a central fear state and fear expression is discussed and how these two areas interact to compose a complete system that is capable of processing and storing negative emotions.

The rodent amygdala is located deep within the medial temporal lobe, adjacent to the hippocampus and lateral to the hypothalamus. The bilaterally located almond shaped structure consists of at least 13 nuclei which are divided into three groups, the basolateral nuclear group, the central-medial group and the medial nucleus (McDonald, 1998). The boundaries of the nuclei are not necessarily clear-cut and often merge with adjacent structures. The areas are distinguishable from each other on the basis of cell and chemical differences. The central amygdala cell structure resembles non-cortical cells similar to those in the striatum (Shammah-Lagnado, Alheid, & Heimer, 2001). Conversely, the basolateral cell structure is more like those in the cortex (McDonald, 1998). The connections supplying information differ between the central and basolateral amygdala. The basolateral amygdala receives sensory information, and is suggested to be involved

in voluntary and classically conditioned emotional behaviours, that pass through the central amygdala whose role it is to express displays of emotions (McDonald, 1998; Shammah-Lagnado, Alheid, & Heimer, 2001). The definition in this thesis of the basolateral nuclear group includes the lateral, basolateral but not the basomedial amygdaloid nuclei, and the central group includes the capsular, lateral, intermediate and medial divisions (Paxinos & Watson, 1998; Pitkanen, Savander, & LeDoux, 1997; Sah, Faber, Lopez de Armentia, & Power, 2003). Figure 1.1 illustrates a simplified relationship between the basolateral, central amygdala and intercalated cell masses, whereby, the central amygdala is medially located with respect to the basolateral amygdala.

Prior research focused mainly on the entire amygdala and specific conclusions were drawn from these studies, but as precision improved it was reported that the different nuclei have different functions within the amygdala, thus creating confusion and sometimes contradictory conclusions. This thesis will discuss the specific roles of the central and basolateral amygdala in relation to fear and fear expression and thus older works exploring the whole amygdala will not be included, but for reviews see (Davis, 1990; Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001)

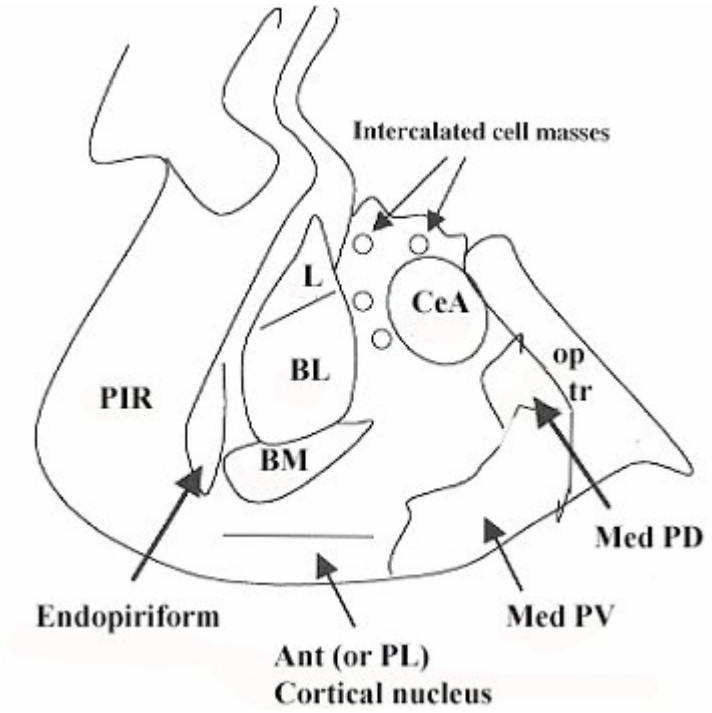

Figure 1.1: Shows the relationship between the basolateral (L, BL), central (CeA) amygdala and the intercalated cell masses. (Pir =piriform, optr=optic tract, med PD-PV=medial dorsal-ventral amygdala, BM=basomedial amygdala).

Neuroanatomy of the central amygdala.

The central amygdala is located in the rostral (front) half of the amygdaloid complex, and may include the substantia innominata and the lateral bed nucleus (Cassell, Freedman, & Shi, 1999). Towards the dorsal surface is the caudate putamen, and ventrally are the intercalated nuclei. It is medially adjacent to the lateral and basal amygdala and laterally adjacent to the stria terminalis. Caudally it is adjacent to the lateral ventricle. The central amygdala can be divided into three areas; the lateral, intermediate and medial divisions. The fourth division, capsular, is sometimes included in the lateral division and is termed the lateral capsular division (Paxinos & Watson, 1998).

Most of the intra-nuclear connections are heavy, unidirectional, and originate in the capsular and lateral (adjacent to the lateral amygdala) and terminate in the medial division of the central amygdala (Pitkanen, Savander, & LeDoux, 1997). Most of these are GABAergic in nature and have been proposed to be inhibitory (McDonald & Augustine, 1993). Some lighter projections exist between the medial and capsular areas but these do not extent to the lateral division. Overall it is a one-way system from the lateral to the medial division. Thus, very few of these connections are reciprocal (Pitkanen, 2001).

Central amygdala afferent and efferent connections.

Afferent connections to the central amygdala arise from numerous brain areas, ranging from the hindbrain to the forebrain. In addition, many afferent connections stem from other amygdaloid nuclei (Pitkanen, Savander, & LeDoux, 1997). The medial division of the central amygdala is the terminal area for most of the inter-amygdaloid connections. Heavy inputs arrive from the lateral, basal, accessory basal, and medial amygdala (Pare, Smith, & Pare, 1995; Pitkanen, Savander, & LeDoux, 1997). Moreover, the intercalated nuclei located adjacent to the basolateral appear to control the effect of basolateral outputs towards the central amygdala (Millhouse, 1986; Pare, Quirk, & LeDoux, 2004; Pare, Smith, & Pare, 1995).

Figure 1.2 depicts the major afferents involved in fear and pain processing. The external inputs arrive from the sub-divisions of the cortex, namely the temporal, insular and parietal cortical regions, and these are notably involved in sensory-related processes (Brunzell & Kim, 2001; McDonald, 1998) and information from the auditory and visual regions (McDonald, 1998). Inputs from the frontal olfactory cortex (sense of smell) and

medial prefrontal cortex mediate fear extinction (Quirk, Likhtik, Pelletier, & Pare, 2003). The hippocampal formation has substantial projections to the central amygdala and is involved in acquisition and fear extinction (Corcoran, 2005). Similarly, the thalamus and the hypothalamus both provide strong inputs and are involved in pain perception (Tanaka, Yoshida, Emoto, & Ishii, 2000). The brain stem, pons, medulla and midbrain including the ventral tegmental and periaductal grey areas all send projections to the central amygdala. Most of these are involved in pain perception, particularly projections from the nucleus parabrachialis (Jasmin, Burkey, Card, & Basbaum, 1997) which is located in the pons (Bernard & Besson, 1990; Davis, Rainnie, & Cassell, 1994). The basal forebrain and striatum (part of the reward system) also connect to the central amygdala but in a lesser way (Shammah-Lagnado, Alheid, & Heimer, 2001). These are not all the connections but are the major afferent connections involved in fear and pain processing.

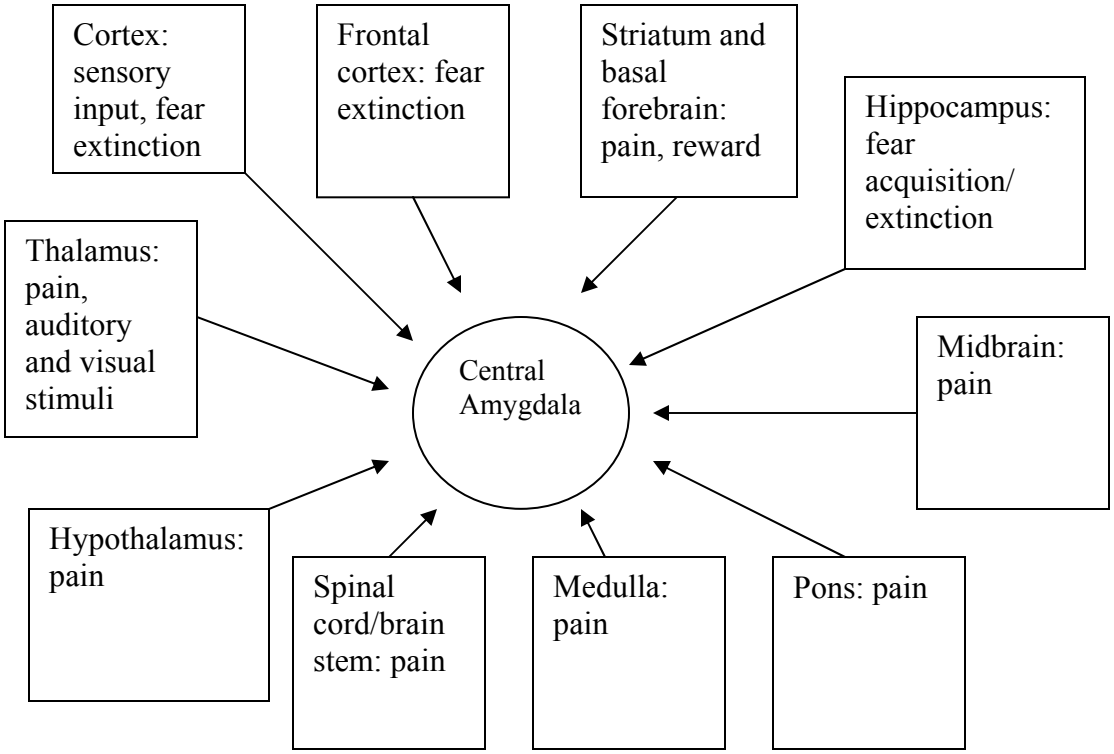

Figure 1.2: Central amygdaloid major afferents involved in pain and fear processing, schematics adapted from Pitkanen (2001).

Figure 1.2 clearly shows that the central amygdala receives both auditory and visual but also pain related information. This suggests a greater role than merely passing information on from the basolateral amygdala to areas that execute fear behaviours.

The prior named connections are not always reciprocal. Figure 1.3 shows that the central amygdala projects to areas related in fear expression, but not to areas involved in receiving external information. For example, the central nucleus does not, appear to project to the olfactory or sensory-related cortical areas, the hippocampal formation or the frontal cortex. Heavy projections are found going to the thalamus, hypothalamus which are involved in heart rate and blood pressure, (LeDoux, Iwata, Cicchetti, & Reis,

1988), basal forebrain, striatum and the bed nucleus of the stria terminalis (Sah, Faber, Lopez de Armentia, & Power, 2003). But most of the projections from the central amygdala arrive in the primitive brain, the midbrain, and periaquaductal gray area, responsible for nociception (Basbaum & Fields, 1984) and freezing behaviour (Brandao, Zanoveli, Ruiz-Martinez, Oliveira, & Landeira-Fernandez, 2008; LeDoux, Iwata, Cicchetti, & Reis, 1988). The central amygdaloid also projects to the pons, in which the nucleus reticularis pontis caudalis is located, that controls the acoustic startle circuit, (Boulis & Davis, 1989; Hitchcock & Davis, 1991; Hitchcock, Sananes, & Davis, 1989; Rosen, Hitchcock, Sananes, Miserendino, & Davis, 1991) and medulla (Sah, Faber, Lopez de Armentia, & Power, 2003). These areas are also collectively responsible for pain perception (Manning, 1998).

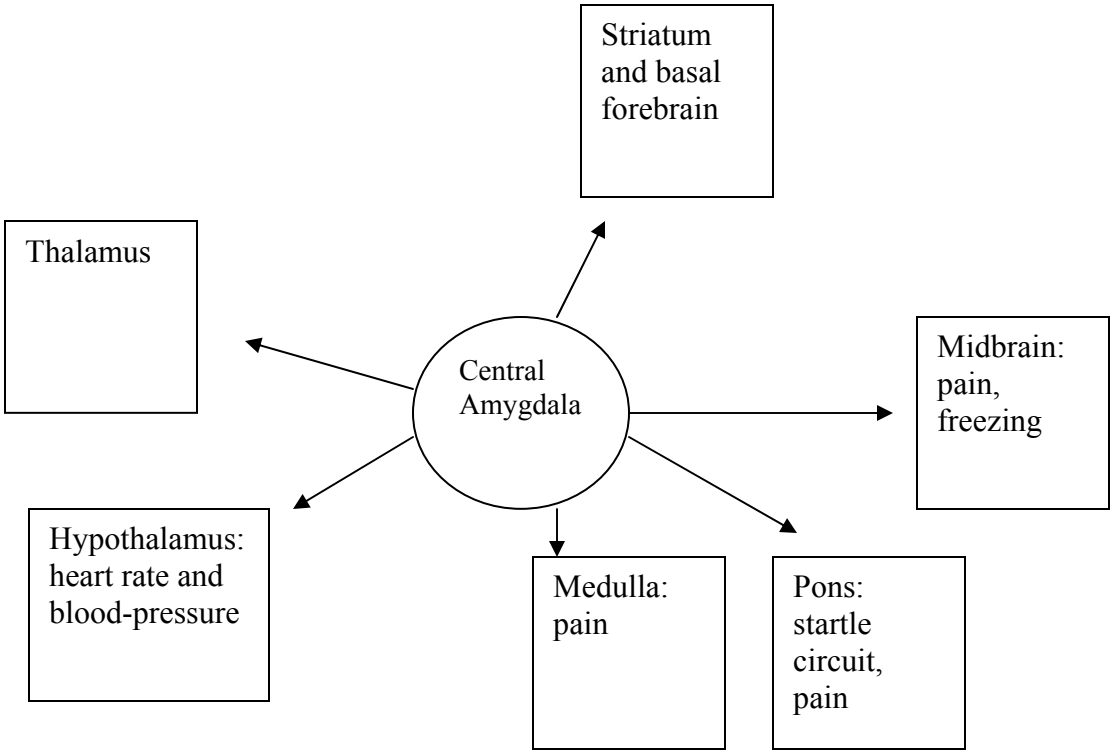

Figure 1.3: Major efferents from the central amygdala involved in pain and fear processing, schematics adapted from Pitkanen (2001).

Most notably the central amygdala receives more information from different areas than that it projects to.

Neurotransmitters affecting central amygdala functions.

Within the central amygdala several types of neural systems have been located. Receptors in these systems respond to a collection of neurotransmitters. The main neurons are GABAergic (McDonald & Augustine, 1993) and these may be configured in a shell-core configuration similar to the nucleus accumbens (Cassell, Freedman, & Shi, 1999). These GABAergic neurons are mainly inhibitory and are affected by muscarinic which is a subtype of the acetylcholinergic receptors and are positively correlated with

fear expression (Roozendaal et al., 1997; van der Zee, Roozendaal, Bohus, Koolhaas, & Luiten, 1997) and anxiety (Sanders & Shekhar, 1995). Whole cell recordings of the lateral division in the central amygdala showed that the $GABA_a$ receptor mediated fast inhibitory transmission. These receptors were affected by benzodiazepines which potentiated the inhibitory effect of GABA receptors (Delaney & Sah, 1999). These results suggest that a GABAergic system in the central amygdala plays an important role in fear and anxiety. Huber, Veinante and Stoop (2005) found that GABA worked in conjunction with the two neuromodulators, vasopressin and oxytocin.

The central amygdala has high densities of vasopressin and oxytocin receptors, which are believed to be involved in autonomic regulation (Veinante & Freund-Mercier, 1997). Vasopressin enhances stress levels and consolidation of fear memories, while oxytocin attenuates these actions (Stoehr, Cramer, & North, 1992). The central amygdala receives nociceptive information which can be regulated via the enkephalinergic opioid receptors (Khachaturian, Lewis, Hollt, & Watson, 1983).

Not only do opioid receptors regulate pain but around 80% of the neurons that respond to noxious stimuli are also affected by glutamate transmission including NMDA and AMPA receptors (Neugebauer, Li, Bird, & Han, 2004; Zhu & Pan, 2004). This nociceptive information is partly governed by noradrenaline (Delaney, Crane, & Sah, 2007) whose precursor dopamine is located in the central amygdala. Here indeed D1 and D2 receptors have been found (Leonard et al., 2003) of which most are D2 –like receptors (Levey et al., 1993). In summary, some of the major neurotransmitters in the central amygdala are GABA, glutamate, and dopamine.

The role of GABA, glutamate and dopamine in fear expression as measured by freezing and startle.

The central amygdala has been reported to be the common pathway from the basolateral amygdala to various centres that control aspects of fear expression (Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001). However, as the following section will show, the central amygdala is also involved in pain perception, shock sensitization, fear acquisition, memory consolidation and fear expression. This is a far more complex role than previously realized.

A central part of fear conditioning is the presentation of shock as an aversive stimulus. At certain levels shock will produce pain; the central amygdala is sometimes refered to as the 'nociceptive amygdala'. Lesions significantly decreased pain sensitivity (Balasubramanian, Pal, & Konar, 2006). Certain cells in the central amygdala become more responsive to nociceptive stimuli (Neugebauer & Li, 2003; Neugebauer, Li, Bird, Bhave, & Gereau, 2003). Li and Neugebauer (2004) were able to block pain expression by the infusion of NMDA and non-NMDA receptor antagonists. Moreover the NMDAr antagonist, AP-5, could block specific noxious stimuli but was not involved in preventing non-nociceptive or background information from stimulating the cental amygdaloid cells. Conversely, the non-NMDAr antagonist CNQX blocked not only specific noxious stimuli but also non-nociceptive and background information. The effects of these agents suggest that pain-related neural mechanisms can be found in the central amygdala. Later studies have shown that this is a post-synaptic effect affecting the nociceptive information from the pontine-parabrachial area to the central amygdala (Bird et al., 2005). The fact that the

central amygdala plays an important role in pain perception has not been fully explored in the study of classical fear conditioning.

Some findings do suggest though that the central amygdala is involved in acquisition and fear expression. For example, lesions of the central amygdala can prevent the expression of shock sensitization (Hitchcock, Sananes, & Davis, 1989), fear potentiated startle (Hitchcock & Davis, 1986) to both visual and auditory stimuli (Campeau & Davis, 1995) and fear expression but not fear reacquisition (Kim & Davis, 1993).

Loud white noise, 120 dB, is aversive for rats and produces freezing behaviour, phasic heart rate accelerations and decelerations, but these are abolished after central amygdala lesions (Young & Leaton, 1996). Likewise, lesions prevent the expression of autonomic responses (Iwata, LeDoux, Meeley, Arneric and Reis, 1986) and hypoalgesia (decreased pain sensitivity) (Helmstetter, 1992) seen during normal fear expression. Conversely, rats showed an increase in vocalization thresholds directly after tail-shock, indicating hyperalgesia (pain enhancement), but lesions to the central amygdala abolished this (Koo, Han, & Kim, 2004). Furthermore, this specific effect was not found in the basolateral amygdala (Crown, King, Meagher, & Grau, 2000). Lesions also modulate conditioned orienting responses to specific cues particularly under increased attentional load, suggesting that the central amygdala may focus attention to fearful objects (Holland, Han, & Gallagher, 2000). Electrolytic lesions attenuated the acquisition of conditioned vocalization and freezing behaviour to tone and context (Goosens & Maren, 2001; Koo, Han, & Kim, 2004; Nader, Majidishad, Amorapanth, & LeDoux, 2001). This

suggests that the central amygdala receives information either about the US or CS or both. This was previously only attributed to the basolateral amygdala.

A difficulty with lesion studies is that it is irreversible and thus making it difficult to establish if it is fibres of passage or actual local damage that cause these deficits. A more precise method is the infusion of a particular drug, of which the effects are reversible, into the area of interest. Analgesic doses of the $GABA_a$ agonist, muscimol, infused into the central amygdala have shown impairments in acquisition of freezing behaviour (Wilensky, Schafe, Kristensen, & LeDoux, 2006) thereby indicating a role for the central amygdala in processing fear-related learning.

The central amygdala contains dopamine neurons and these are reported to enhance attention to cues (Gallagher & Holland, 1994). Interestingly, a D1 agonist, SKF 82958, infused prior to acquisition can enhance freezing behaviour during testing, conversely the infusion of the dopamine D1 antagonist (SCH 23390) prior to acquisition can prevent fear learning (Guarraci, Frohardt & Kapp, 1999). Similarly, the infusion of a D2 antagonist, eticlopride, prior to acquisition also reduced conditioned freezing responses to an explicit CS and to contextual cues (Guarraci, Frohardt, Falls and Kapp, 2000). These results indicate that dopamine plays a role in acquisition and this has mostly been attributed to changes in attention to specific stimuli (Gallagher & Holland, 1994). Thus both GABA and dopamine neurotransmission appear to influence acquisition. It is not clear if this was due to the inability to experience the fear state or if it was a specific deficit in attention to salient cues. The roles of these neural systems have received little attention during fear expression to US or to CS.

Fear acquisition can be affected by glutamate receptor inhibition (Goosens & Maren, 2003). The NMDAr inhibitor AP-5 infused prior to conditioning resulted in attenuated freezing responses to both context and explicit CS on test day. Furthermore, the rate of reacquisition was enhanced indicating a savings on later acquisition tests (Goosens & Maren, 2003). Savings on learning indicate, that it takes less time to learn the task because there is residual memory and this suggests a potential for the central amygdala to be involved in long term potentiation. In vitro studies support the idea that the central amygdala is involved in a different form of NMDA-dependent long-term potentiation (Samson, Duvarci, & Pare, 2005; Samson & Pare, 2005). Moreover, the infusion of the protein synthesis inhibitor, anisomycin, severely affects memory consolidation (Wilensky, Schafe, Kristensen, & LeDoux, 2006). This again, suggests that a type of synaptic plasticity in the form of long-term potentiation takes place in the central amygdala. Thus the central amygdala may mediate forms of fear acquisition.

In previous research it became clear that the central amygdala has a role in fear expression. Walker and Davis (1997) reported that infusions of the AMPAr antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(F)-quinoxaline) can block the expression of fear-potentiated but not light-enhanced startle. Light-enhanced startle was suggested to be a measure of an unconditioned response similar to anxiety (Davis, 1998). Exposing rats to bright lights would enhance startle but this was not affected by NBQX infusion (Walker & Davis, 1997b). Likewise, the expression of anxiety can be reduced by the infusion of muscimol, a $GABA_a$ agonist, into the central amygdala. Anxiolytic effects of this agent were found in a social interaction test, during which hamsters showed reduced anxiety when confronted by a socially dominant conspecific. Conversely,

injecting bicuculline a $GABA_a$ antagonist had no effect (Sanders & Shekhar, 1995). Central amygdala inactivation via the infusion of the $GABA_a$ agonist muscimol prevented the expression of freezing behaviour (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

In summary, lesions of the central amygdala prevent the expression of shock sensitization and fear-potentiated startle. Temporary inactivation reduces the expression of freezing behaviour. In addition, non-NMDA and dopamine D1 receptor antagonism also prevent fear expression. These outcomes suggest that the central amygdala should be explored to further understand its function in fear expression after the presentation of a US (foot-shock) during the shock sensitization paradigm and compare this to the results of fear expression to an explicit CS. In this thesis the GABAergic system was probed by the infusion of a low dose of muscimol, and the glutamatergic system by an NMDA and an AMPA antagonist. The dopaminergic system was challenged by the infusion of a D1 agonist or a D2 agonist. Finally, during both paradigms, rats were infused with the protein synthesis inhibitor anisomycin. All of the outcomes support a particular role for the central amygdala in fear expression. Specifically, glutamate contributes to pain processing and dopamine to attention to cues.

Neuroanatomy of the basolateral amygdala.

This chapter introduces the neuroanatomy and the function of the basolateral amygdala, with a focus on the rat brain, in relation to fear processing as measured by acoustic startle and freezing behaviour.

The basolateral or deep amygdaloid nuclei are located within the temporal lobe and are partitioned into the lateral, basal and accessory basal nucleus, (Pitkanen, Savander, & LeDoux, 1997). However, terminology can differ between authors; sometimes the lateral and the basal nucleus are called the basolateral amygdalar complex (Koo, Han, & Kim, 2004) and the accessory basal nucleus the basomedial nucleus. Although the lateral and basal nuclei may have subtle functional differences, these areas are often treated as one, (Koo, Han, & Kim, 2004). For example, no significant functional differences between the lateral and basal nuclei were found when examining the major excitatory NMDA receptors in their effect on fear expression (Lindquist & Brown, 2004). The studies conducted in the present thesis were aimed at understanding the function of the lateral and basal nuclei and did not include the basomedial area.

Basolateral amygdala afferent and efferent connections.

The lateral amygdala is located in the upper or dorsal section of the amygdaloid complex, adjacent to the medially located central amygdala at the rostral location and adjacent to the lateral ventricle towards the caudal location. Towards the ventral side it is adjacent to the basal amygdala. Most of the connections within the lateral amygdala are unidirectional going from dorsal to ventral and dorsal to medial divisions (Pitkanen, Savander, & LeDoux, 1997).

Intra-amygdaloid connections show that the lateral division receives heavy projections from the ventrally located basal, accessory basal and medial nuclei; this is a bidirectional relationship (Savander, Miettinen, LeDoux, & Pitkanen, 1997). Additionally, the lateral amygdala projects to the central nuclei, but this does not appear to be reciprocal (Pitkanen, Savander, & LeDoux, 1997).

The basal nucleus is ventral to the lateral nuclei and comprises 3 subdivisions - the magnocellular, intermediate and parvicellular divisions. The basal nucleus is between the laterally placed external capsule and the central amygdala towards the rostral end, but between the external capsule and the lateral ventricle towards the caudal end. Caudal and rostral projections originate mostly in the parvicellular division and project to the intermediate division and are mainly reciprocal. The parvicellular division is responsible for most of the inter-amygdala connections, specifically to the lateral division, which is reciprocal. Thus, information is reciprocal between the lateral and basal amygdala and both nuclei project towards the central amygdala. The projections to the central amygdala pass through the intercalated cell masses (Millhouse, 1986). Stimulation in the BLA can produce inhibitory responses in the central amygdala via these, and modulation by the intercalated masses affects the amount of inhibition produced (Pare, Quirk, & LeDoux, 2004; Sah, Faber, Lopez de Armentia, & Power, 2003).

Connections to and from cortical areas do not differ greatly between the lateral and basal amygdala. Figure 1.4 illustrates the afferent and efferent neural connections in relation to the basolateral amygdala. Please note that neither the midbrain nor the pons show reciprocal connections.

The basolateral divisions have reciprocal connections with the pre-frontal cortex and the medial temporal lobe system or limbic system. These anatomically related areas contain the olfactory and hippocampal formation. The basolateral divisions have light reciprocal connections with the olfactory system. But, reciprocal information from the hippocampus to both areas is substantial. Heavy projections innervate this area which is primarily responsible for episodic and declarative memory formation and spatial navigation. Evidence suggests that the hippocampus mediates fear acquisition and consolidation (Sanders, Wiltgen, & Fanselow, 2003).

The basolateral areas have strong reciprocal relationships with the thalamus which is responsible for organising sensory information, motor systems and arousal. Notably, it is involved in fear expression to auditory stimuli (Heldt & Falls, 1998). The neurons in the lateral amygdala also respond to auditory and somatosensory stimuli sent via the thalamus (Romanski, Clugnet, Bordi, & LeDoux, 1993). The lateral amgdala area is believed to be the location for the convergence of CS and US associations (LeDoux, 2000). The hypothalamus which is more involved with endocrine functions has only light connections to the basolateral amygdala.

Furthermore, both the lateral and the basal divisions receive light connections from the striatum (planning and movement, but also intense unexpected movements) but both have heavy outputs towards the striatum. Lesioning the dorsal striatum, for example, reduces fear conditioning to explicit cues but not to contextual cues (Ferreira, Moreira, Ikeda, Bueno, & Oliveira, 2003).

Some subtle differences between the lateral and basal divisions are that the lateral division, unlike the basal division, receives inputs from sensory areas such as the

temporal, insular and parietal cortices. These areas are involved in sending visual, somatosensory, gustatory, auditory and visceral US information to the lateral amygdala (McDonald, 1998; Rodgers, Benison, Klein, & Barth, 2008; Shi & Davis, 1999). Both the lateral and the basal division reciprocate by sending information to these sensory areas. Both the midbrain and the pons send information to the basolateral area, particularly information concerning stress (Inglis & Moghaddam, 1999). The ventral tegemental and the pariaquaductal gray areas both located in the midbrain are involved in defensive behaviour, for example freezing, but also in fear conditioning (Gelowitz & Kokkinidis, 1999). Finally the basolateral group has major projections to the central amygdaloid complex, but these projections appear to be unidirectional (Pare, Smith, & Pare, 1995).

Essentially, the basolateral amygdala is a neural hub that sends and receives information and modifies this according to the type of input (Pitkanen, Savander, & LeDoux, 1997). The lateral and basal amygdalae are mostly investigated as a single area. However, the lateral amygdala has received attention specific to CS-US acquisition (Amorapanth, LeDoux, & Nader, 2000).

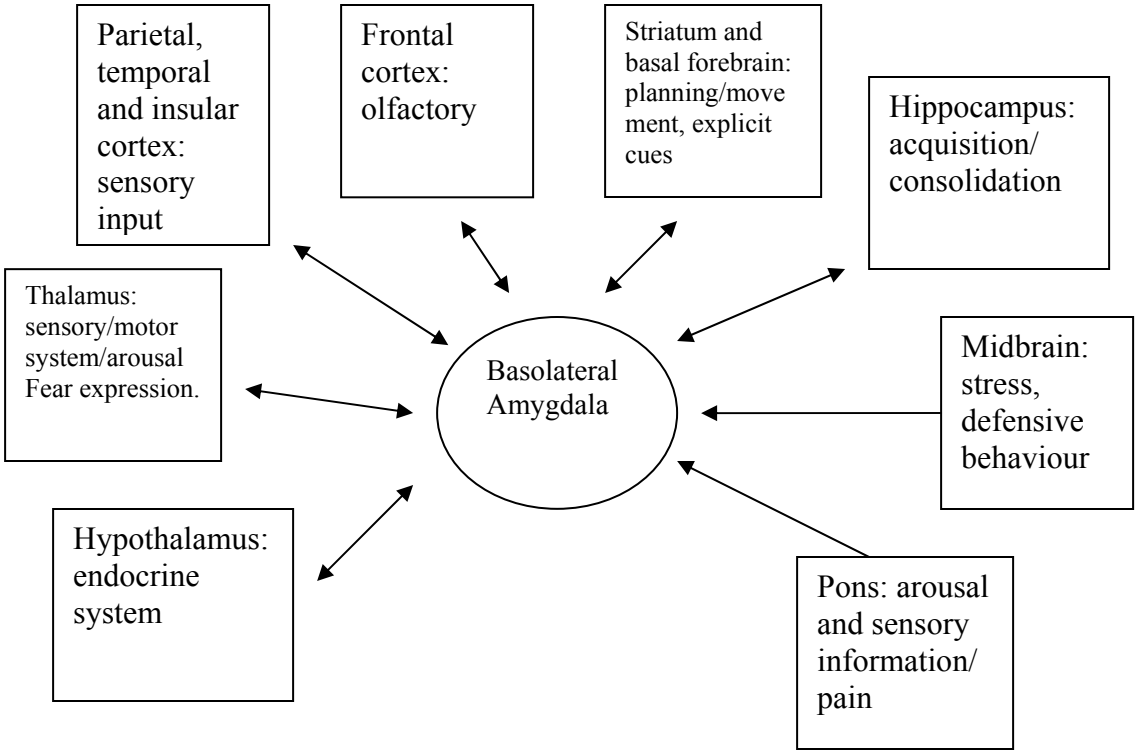

Figure 1.4: Afferent and efferent connections in relationship to fear behaviour and pain in the basolateral amygdala. The schematics have been adapted from Pitkanen (2001).

Neurotransmitters affecting basolateral functioning.

The major neurotransmitters examined in this thesis were GABA, glutamate and dopamine. The main amino acid-based neurotransmitters found in the basolateral amygdala are glutamate and GABA, of which the inhibitory receptors are GABAergic and the excitatory receptors are glutamatergic. Dopamine can either have an inhibitory or excitatory function depending on its location on the neuron, of which the D1 like receptors are excitatory and the D2 like receptors inhibitory.

The GABAergic system in the basolateral amygdala contains interneurons that effectively inhibit synaptic potentials (Sah, Faber, Lopez de Armentia, & Power, 2003). This can either be via a feedforward system involved in information received from the

cortical and thalamic tracts, or by a feedbackward system that is activated by information arriving from the cortical tracts (Dityatev & Bolshakov, 2005; Woodruff, Monyer, & Sah, 2006). Thus, depending upon the incoming information, the GABAergic system can have a range of firing patterns. These firing patterns can effectively inhibit glutamatergic excitatory postsynaptic potentials (Dityatev & Bolshakov, 2005; Woodruff, Monyer, & Sah, 2006).

Between 85-95% of the basolateral cells projecting to the prefrontal cortex are responsive to glutamate (McDonald, 1996). In addition, major cortical and thalamic inputs are also glutamatergic in nature (Farb & LeDoux, 1997). These are responsive to NMDA, AMPA and kainate stimulation, and are excitatory. Both NMDA and AMPA receptors are present at synapses in these neurons, of which AMPA receptors are mostly activated earlier than NMDA receptors (Dityatev & Bolshakov, 2005; Watt, Sjostrom, Hausser, Nelson, & Turrigiano, 2004). However, depending upon the direction from which incoming information arrives, NMDA receptors can be activated first due to lower levels of magnesium that block the receptor (Sah, Faber, Lopez de Armentia, & Power, 2003) thereby indicating that, during the resting potential of the neuron, NMDA receptors can be activated under certain circumstances. For example, thalamic neurons have a smaller AMPA/ NMDA ratio on the dendrites of the neuron suggesting a greater contribution of NMDA receptors and are thus more easily activated (Weisskopf & LeDoux, 1999).

Dopamine has a neuromodulating function and is capable of either inhibitory or excitatory functions (Missale, Nash, Robinson, Jaber, & Caron, 1998; Rosenkranz & Grace, 1999; Tamminga, 2005). Both the D1 and the D2 receptor are widely found in the

basolateral amygdala (Boysen, McGonigle, & Molinoff, 1986; Kroner, Rosenkranz, Grace, & Barrionuevo, 2005; Young & Rees, 1998), and dopamine has been shown to directly change the excitability of basolateral amygdala neurons (Kroner, Rosenkranz, Grace, & Barrionuevo, 2005). D1 or D2 receptor activation leads to potentiation of the strongest sensory input via an increase in firing rate of the fast firing neurons while attenuating the slow firing neurons (Kroner, Rosenkranz, Grace, & Barrionuevo, 2005; Rosenkranz & Grace, 1999). The production of dopamine and norepinephrine are closely related since dopamine is the precursor for norepinephrine (Delcomyn, 1998). The noradrenergic system is controlled by norepinephrine which is released notably during stressful situations (Galvez, Mesches, & McGaugh, 1996). Together these results show a potential role for the dopaminergic/ noradrenergic system in the basolateral amygdala.

The role of GABA, glutamate and dopamine in the basolateral amygdala during fear processing.

One of the primary ways for investigating a brain area is to lesion it via electrical or chemical means. Electrolytic lesions destroy the area including fibres of passage while chemical lesions can spare these, depending upon the type of chemical used. In the basolateral amygdala, information concerning the US and CS assemble and become associated here (Goosens & Maren, 2001; Romanski, Clugnet, Bordi, & LeDoux, 1993; Shi & Davis, 1999). By default this implies that lesioning must prevent the association and thus the expression of the association.

Lesioning the basolateral amygdala can produce a variety of behavioural deficits. A significant finding is that shock sensitization is abolished by NMDA lesions, while foot-shock induced activity remains unaffected (Sananes & Davis, 1992). Thus the function of

foot-shock to elicit fear is eliminated. Therefore it would not be unexpected that lesions of the basolateral amygdala before training would eradicate the expression of freezing behaviour (Nader, Majidishad, Amorapanth, & LeDoux, 2001). And the effect of pretraining neurotoxic lesions can be attenuated by overtraining whereby, over time, rats will show significant freezing to context but not to an explicit CS (Maren, 1999b). This indicates that potentially alternative routes exist for the acquisition of the relationship between the context, explicit cues and US.

Lesions of the basolateral amygdala after training disrupts the expression of freezing behaviour (Maren, 1999b; Maren, Aharonov, Stote, & Fanselow, 1996) and fear potentiated startle (Sananes & Davis, 1992). A lesioned basal nucleus has no influence on acquisition but does attenuate the expression of fear as measured by freezing behaviour (Anglada & Quirk, 2005). These lesion studies show that the basolateral amygdala affects the expression of fear to a US but also to a CS. Moreover, not only the basolateral amygdala processes US/CS information but perhaps areas such as the central amygdala are involved as well.

Lesions cause irreparable damage and can destroy fibres of passage. This makes it difficult to delineate if it is the area itself that is responsible for the effect or if this is caused by destruction of the fibres that pass throught here. Exploration of an area via reversible temporary inactivation is a more refined method. For example, temporary inactivation of the basolateral amygdala with muscimol impaired the acquisition and expression of freezing behaviour to both contextual and explicit conditioned stimuli (Helmstetter & Bellgowan, 1994; Huff & Rudy, 2004; Muller, Corodimas, Fridel, & LeDoux, 1997; Wilensky, Schafe, & LeDoux, 1999). This indicated that the basolateral

amygdala has an important role in fear expression to CS presentation. Because acquisition is also prevented, it is plausible that this is either a memory or a US processing deficit. Currently, no specific studies have been undertaken to understand the effect of muscimol on the expression of fear on presentation of an US. An often used $GABA_a$ agonist, muscimol, increases the inhibitory function of the GABAergic system and functions as a local anaesthetic. By reducing its concentration it is possible to clarify if the actual GABA neuromechanism is involved. Currently, no studies have been carried out to determine if small doses can suppress fear expression.

The glutamate system consisting of NMDA and AMPA/kainate receptors have multiple roles in the basolateral amygdala. A function of AMPA receptor antagonism is that it prevents excitatory action potentials thus inhibiting the transfer of information between neurons (Delcomyn, 1998). The AMPA receptor antagonists, CNQX and NBQX have both been shown to block fear-potentiated and light enhanced startle to a specific CS (Walker & Davis, 1997a). During light enhanced startle, rats were exposed to bright light followed by an acoustic startle probe; the augmented startle was interpreted as an anxiety response. Additionally, NBQX can prevent fear-potentiated startle to an odor but not to context (Walker, Paschall, & Davis, 2005). Together, these results show a specific role for AMPA neurotransmission in fear expression to explicit cues, but so far no information is available on the function of AMPA receptors during fear expression to a US.

NMDA receptors have been primarily implicated in long term potentiation (Rogan, Staubli, & LeDoux, 1997b) and thus synaptic plasticity which is the cornerstone of learning (Abel & Lattel, 2001; Dityatev & Bolshakov, 2005). During long-term

potentiation neuronal changes occur that require coherent team work between AMPA and NMDA receptors. Once these receptors are activated, biochemical cascades within the postsynaptic neuron happen, which involve structural changes. These changes can be interrupted by various enzymes and protein synthesis blockers (Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006). One of these is anisomycin (Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999). However, NMDA receptors are not exclusively used for long-term potentiation but also for normal synaptic transmission (Li, Inoei, Abekawai, YiRui, & Koyama, 2004; Weisskopf & LeDoux, 1999). Thus, multiple roles for NMDA neurotransmission exist.

For example, the infusion of the NMDA antagonist AP-5 can either enhance fear potentiated startle over and above normal potentiation (Gewirtz & Davis, 1997) or block the expression of fear as measured by freezing (Fendt, 2001; Lee & Kim, 1998; Lee, Choi, Brown, & Kim, 2001; Maren, Aharonov, Stote, & Fanselow, 1996) and fear-potentiated startle (Fendt, 2001). Other researchers have shown no effects to small (1.25 µg/µl AP-5, Miserendino, Sananes, Melia, & Davis, 1990) and medium sized dose rates (2.5µg/µl AP-5, Campeau, Miserendino, & Davis, 1992). The discrepancies noted above could be due to differences in paradigms and cannulae locations (Fendt, 2001). An alternative explanation is that NMDA receptors participate in normal neural transmission (Li, Inoei, Abekawai, YiRui, & Koyama, 2004; Weisskopf & LeDoux, 1999). For example, AP-5 diminished the expression of ultra-sonic vocalization, defecation and analgesia after conditioning to context or tone cues. These were suggested to accrue from deficits in normal neurotransmission and are not long-term potentiation dependent (Lee, Choi, Brown, & Kim, 2001).

The development of associations between the CS and US and second-order conditioning are blocked by the infusion of AP-5, suggesting that NMDA receptors are also involved in neural plasticity (Campeau, Miserendino, & Davis, 1992; Gewirtz & Davis, 1997; Lee & Kim, 1998; Maren, Aharonov, Stote, & Fanselow, 1996). Additionally, rats trained under the influence of AP-5 could not express freezing behaviour to contextual or explicit stimuli (Goosens & Maren, 2004). These rats showed no savings during further acquisition suggesting that during initial training under AP-5 influence, no memory traces were laid down that could augment further learning. Similarly, Syrian hamsters also failed to acquire conditioned defeat after AP-5 infusion (Jasnow, Cooper, & Huhman, 2004). Furthermore, the extinction of acquired fear was blocked by AP-5 (Lee & Kim, 1998). Extinction is proposed to represent the formation of new associations between the previously acquired CS_1 and a novel CS_2. This can be achieved via the omission of the US after CS_1 presentation. Together these results suggest a definite role for NMDA neurotransmission in fear acquisition.

Analysing the role of glutamate Rodrigues, Bauer, Farb, Schafe, and LeDoux (2002) found that the group 1 metabotropic receptor subtype, mGluR5, is required for acquisition and long-term potentiation of freezing behaviour, but has no influence on expression. Conversely, infusion of a subtype of the metabotropic group 2 could prevent expression (Lin, Lee, Huang, Wang, & Gean, 2005). The overall consensus is that NMDA receptors are critical for fear responses either via a reduction in overall excitatory neurotransmission (Li, Inoei, Abekawai, YiRui, & Koyama, 2004; Weisskopf & LeDoux, 1999) or via interference during long-term potentiation (Watt, Sjostrom, Hausser, Nelson, & Turrigiano, 2004). Even though NMDA is implicated in long-term potentiation and

excitatory neurotransmission, and appears to inhibit fear expression to a CS, no information is available concerning NMDA receptors involvement in fear expression to US presentation.

Neural plasticity induced during long-term potentiation requires protein synthesis (Stork, Stork, Pape, & Obata, 2001), which can be disrupted by the infusion of various mRNA synthesis inhibitors such as Actinomycin-D (act-D) (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999) and the protein synthesis inhibitor anisomycin (Schafe & LeDoux, 2000). Bailey, Kim, Sun, Thompson, and Helmstetter, (1999) reported that infusion of act-D temporally disrupted the acquisition of freezing behaviour to context and an explicit CS. Rats that were trained under act-D influence failed to show freezing behaviour, but could do so a week later after more training suggesting that act-D has a temporary effect and disrupts long-term potentiation. Anisomycin infused after conditioning reduced expression of freezing behaviour to explicit and contextual cues, but not when infused prior to conditioning (Huff & Rudy, 2004). This indicated that structural changes affected by anisomycin occurred post training. Additionally, recall of a memory can make that memory labile (Nader, Schafe, & LeDoux, 2000a). These authors infused anisomycin into the BLA of rats before consolidated-memory retrieval and found that rats could show freezing behaviour to explicit CS but failed to show this 24 h later. It is unclear if this was a failure of the activation of memory traces or if it affected the central fear state per se. Again no research has been completed on the function of anisomycin during fear expression to US or to CS.

The neural systems controlled by dopamine have been investigated mainly with the use of D1 and D2 antagonists infused throughout the whole amygdala, thereby

confounding interpretations. However, bilateral infusion into the basolateral amygdala of the dopamine D1 antagonist, SCH 23390, did not interfere with shock reactivity and shock sensitization but did attenuate the acquisition of fear potentiated startle (Greba & Kokkinidis, 2000). Similar effects were found after infusion of the D2 antagonist raclopride (Greba, Gifkins, & Kokkinidis, 2001). Thus dopamine antagonists prevented fear acquisition but not through a suppression of shock-associated fear activation. A closely related neurotransmitter that sometimes affects the same neural system as dopamine is norepinephrine. Norepinephrine has also been implicated in expressions of fear and stress. For example, the reduction of norepinephrine by clonidine injections could block acquisition and expression of fear potentiated startle (Schulz, Fendt, & Schnitzler, 2002). Norepinephrine release is increased during stressful situations particularly under foot-shock application (Galvez, Mesches, & McGaugh, 1996; Quirarte, Galvez, Roozendaal, & McGaugh, 1998; Tanaka, Yoshida, Emoto, & Ishii, 2000), immobilization stress (Tanaka, Yoshida, Emoto, & Ishii, 2000), and is involved in regulating long-term memory storage (Clayton & Williams, 2000; Ferry, Roozendaal, & James, 1999). The infusion of a noradrenalin agonist, clonidine, affects not only fear acquisition but also fear expression (Schulz, Fendt, & Schnitzler, 2002). Even though the nor-adrenergic system is not explored in this thesis it is still possible that the dopamine system may overlap with the adrenergic system, because dopamine is the precursor for norepinephrine (Delcomyn, 1998).

Little is known about the effects of dopamine agonists on the effect of US-and CS-provoked fear expression. Thus, it is important to explore the role of D1 and D2 agonists on fear responses during the presentations of US and CS.

The interactions between the central and basolateral amygdala.

As shown in the preceding sections, both the central and basolateral amygdala perform a definite role in fear expression to presentation of an US or CS. Since these nuclei are adjacent to each other, various models of neurotransmission have been proposed. The earlier models suggested that the central amygdala was used as a 'pathway' for the information that was collated in the basolateral amygdala (Fendt & Fanselow, 1999). The information was relayed in a serial manner, thus from the basolateral amygdala to the central amygdala. However, as has already been shown, the central amygdala plays a more important role than merely as a 'pathway'. An alternative representation to the serial model is a parallel model (Pare, Quirk, & LeDoux, 2004). In this model both the central and basolateral amygdala receive US and CS information. This is of particular importance because the central amygdala is receptive to pain. Since foot-shock elicits a reflex, pain and a central fear state (Borszcz, 1993, 1995), it is important to understand how this information is distributed in the amygdala.

Function of the intercalated cells.

As reported previously, information transfer between the basolateral amygdala and central amygdala appears to be uni-directional, travelling from the former to the latter (Pitkanen, Savander, & LeDoux, 1997). This 'one-way' system introduces a variety of problems, particularly since the central amygdala is deemed the nociceptive centre and the lateral amygdala is the site for the association between the CS and US (Romanski, Clugnet, Bordi, & LeDoux, 1993). Pain information received in the central amygdala from, for example foot-shock, may be necessary for effective CS/US associations. This means that information from the central amygdala must flow 'backwards' to the lateral

amygdala. Or perhaps the central amygdala is also a site for synaptic plasticity where US/CS information becomes associated. Structures that have not received much attention and that are situated between and around the amygdaloid nuclei are the intercalated cells (see Figure 1.1). These cells have failed to attract much interest because they are found in small clusters of thin sheets, thus being difficult to study with the more common laboratory in vivo techniques. However, Golgi preparations show that the intercalated cells form a net that wraps around the various amygdaloid nuclei and thus most likely mediate information flow between the basolateral and central amygdala (Millhouse, 1986). These cells show inhibitory and excitatory characteristics mediated by GABAergic and glutamatergic systems.

In 2004, Paré, Quirk and LeDoux suggested a new organization within the amygdala. They proposed that the older more conventional organization could not explain all that has been more recently discovered about the amygdala. The older model suggested that CS and US inputs from the thalamus converged at the lateral amygdala which is the critical site for neuroplasticity (Romanski, Clugnet, Bordi, & LeDoux, 1993). From this point the information is transferred to the central amygdala which has always been seen as a passive relay. While notably controlling fear expression via the periaductal grey area for freezing behaviour, and the pontine reticular formation for fear-potentiated startle, the central amygdala is also responsible for pain perception. So to acquire fear through Pavlovian conditioning, it seems likely that the information must come through the central amygdala first and then converge at the lateral amygdala. Unfortunately, information from the central amygdala is unable to reach the basolateral

amygdala because of the lack of bidirectional fibres. To date, only unidirectional fibres have been found going from the lateral to the central amygdala (Pitkanen, 1997).

The new model proposes that CS and US information from the thalamus is transferred to both the lateral and central amygdala (Lanuza, Nader, & LeDoux, 2004). This appears logical because the central amygdala receives nociceptive information from the parabrachial area in the brain stem and the thalamus. Paré et al. (2004) suggested that the central amygdala is capable of synaptic plasticity and thus may store a representation of the US-CS connection. It does this via stimulation of the lateral amygdala which in turn dis-inhibits the intercalated cells that are found between the basolateral group and the central group. This unidirectional pathway is controlled by glutamate receptors which in turn activate GABAergic cells that dis-inhibit cells in the medial central amygdala thereby allowing for the information about the US and CS to converge via activity-dependent plasticity in the central medial amygdala. In vitro studies have shown that the basal and lateral amygdala nuclei inhibit action in the central medial amygdala through the intercalated masses (Rosenkranz, Buffelalari, & Grace, 2006). Perez de la Mora et al. (2005) proposed a potential neuronal mechanism for this, by infusing the D1 antagonist SCH 23390 into the rostral intercalated islands they reduced anxiety in rats during a white/black preference test. They postulated that the D1 receptors may attenuate the GABA-mediated dis-inhibition in the central amygdala. It now appears that the central amygdala is not only implicated in fear expression but also in acquisition and consolidation of fear associations (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

Research questions:

This thesis aimed to explore the differences and similarities between fear produced by foot-shock (US) exposure and fear produced by a conditioned stimulus (CS). It was felt that some of the features produced by footshock would also be noted during CS presentation. Specifically it was postulated that the central fear state would be a similarity and higher cognitive functioning would be a difference.

This possibility was tested by investigating the role of the basolateral amygdala. Rats with cannulae aiming for this area were infused with neurotransmitters that have been shown to be involved in components of fear expression. The neural systems explored were those characterised by activity of GABA, glutamate, dopamine and protein synthesis.

Secondly, the significance of the central amygdala was targeted, based on the knowledge that pain is processed in these nuclei. Thus it was important to determine if fear produced by foot-shock was the same as fear produced by a CS. Again differences and similarities were expected. Rats with cannulae aiming for the central amygdala were infused with the same neurotransmitters as used for the basolateral groups. This was done so that it was possible to compare results both within and between each area.

METHOD

Procedures and equipment

Subjects

Male albino Wistar rats bred at the University of Canterbury served as subjects. They were group housed, 4 rats per cage, with free access to water and food. When their weight reached between 350 and 400 grams they were selected for surgery. Testing occurred during the light portion of the light-dark cycle, light on at 0800 off at 2200. All procedures were in strict compliance with local and national guidelines as approved by the Animal ethics committee of the University of Canterbury. Numbers were kept to a minimum to comply with the latest national guidelines.

Surgery

Each rat was anaesthetized by intraperitoneal (IP) injection of a combination of xylazine (5.0 mg/kg) and ketamine (50.0 mg/kg). The plane of anaesthesia was initially monitored by pinching the tip of the tail and later by the movement of the vibrissae. If the rat showed any movement of the vibrissae during surgery it received an extra IP injection of 0.3ml xylazine/ ketamine (5.0 mg/kg, 50.0 mg/kg). Once fully anaesthetized the rat's head was shaved and the skin cleaned with a chlorohexidine solution. Analgesia (norocarp, 0.1ml subcutaneously and mepivicaine 0.1ml local anaesthetic) was administered prior to mounting the skull between the ear-bars. Once the skull was mounted in a stereotaxic (Stoelting, Wood Dale, IL, USA), using ear and incisor bars, an incision was made in the anterior/posterior plane. The skull was exposed and skin was

held back by haemostats. Lamba and Bregma were marked, and the skull levelled. The incisor bar was raised or lowered until dorso-ventral measurements at lambda and bregma were equal.

The appropriate coordinates extrapolated from Paxinos and Watson (1998), for the Bla were AP., -3 ML., ± 4.9 DV., -8.3, and for the Ce AP, -3, ML., ± 4.7, DV., -8.2 were marked with a marking pen and predrilled ready to receive the cannulae (C313G; Plastic One, Roanoke, VA, USA). Four small burr holes were drilled so stainless steel jewellers' screws (3.20 mm long, Lomat Precision Tools Quebec Canada) could be screwed into place. Screws and cannulae were inserted, and held permanently in place with dental acrylic. The wound was sutured and dummy stylets (C313DC; 28 gauge; Plastic One, Roanoke, VA, USA) were inserted in the cannulae to maintain patency.Rats were placed on a heat-mat to recover and returned to a clean post-surgery cage. The rats were group housed post-surgery, 4 rats per cage. Experimental procedures began 5-7 days after surgery.

Infusion.

All drugs (Sigma-Aldrich, Australia) were either freshly prepared or defrosted less than 30 days after being kept in -18 degree Celsius storage. The stainless steel infusion cannulae attached to polythene tubing (PE 20, Plastic One Roanoke, VA, USA) were carefully preloaded and tested for flow. Once inserted into the guide cannulae the tip of the infusion cannulae extended at most 1mm below the guide cannulae. The polythene tubing was attached to two, 2µl Hamilton syringes (Hamilton Co., Reno, Nv, USA) driven by a modified Stoelting infusion pump (Model 310; Stoelting Co. Wood Dale, IL, USA).

Drugs were infused at a volume of 0.5 µl at a flow rate of 0.5 µl/min and the infusion needles were left in place for a further 2 minutes to allow diffusion of the drug to occur. Rats were hand-held during the infusion and then placed in the testing chambers for 5 minutes prior to testing.

Perfusion and histology.

The rats were injected IP with a lethal dose of sodium pentobarbitone and perfused intracardially with 0.9% saline followed by a 10% formalin solution. Their brains were removed and stored in the 10% formalin solution for 2 days. The formalin was then replaced with a 70% sucrose solution and the brains were left to cure for more then 3 days.

Coronal sections of 50 µm were cut on a cryostat (OTF Bright Instrument Company, Huntingdon, England) and placed on prepared gelatine coated glass slides. The sections were stained with 0.5% cresyl violet solution and viewed under a light microscope to verify the cannulae locations.

Since this research investigated the differences between the basolateral and central amygdala, data from rats with cannulae placed outside these regions (including the intercalated cell islands) were rejected.

Below are photographic representations of cannulae placements for both the basolateral and central amygdala.

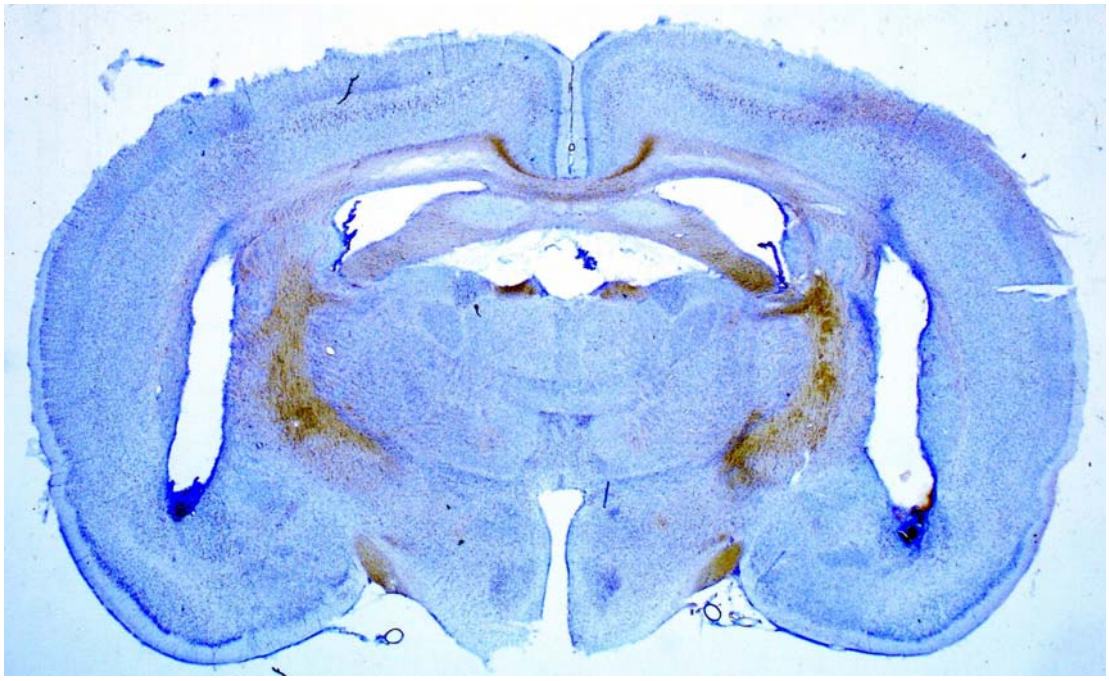

Figure 2.1: Representation of guide cannulae placed in the basolateral amygdala.

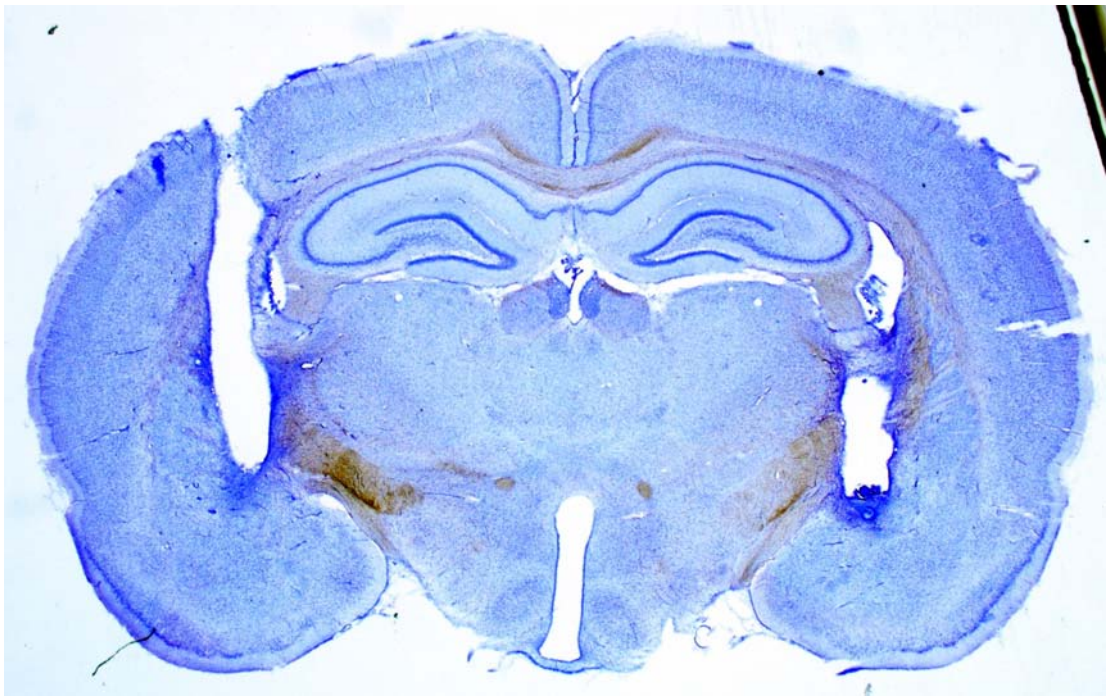

Figure 2.2: Representation of guide cannulae placed in the central amygdala.

Apparatus.

Two sets of testing chambers were used. They provided two different contextual environments. In chambers #1, the rat holding cages had smooth plastic floors and fine wire mesh walls. Conversely, in chambers #2 the rat holding cages had stainless steel bars for floors and walls. All rat holding cages were in sound and light attenuating chambers. Chambers #1 were only used for one behavioural experiment and only to provide an acoustic startle base-lining manipulation. All other manipulations were conducted in chambers # 2.

Acoustic startle chambers # 1: PING Boxes (locally designed)

For one of the behavioural studies, these four identical rat holding cages (150mm x 95mm x 100mm) were used, each suspended 4 cm above a Piezo-electric film which was covered with insulation foam and a mylar (plastic) sheet. The cages were placed in sound attenuating Styrofoam chambers (330mm x 260mm x 270mm). The change in voltage amplitude was measured via a Piezo-electric film that responds to mechanical stress such as kinetic movement produce by a rat. The corresponding voltage change was recorded and 500 mV peak voltage equaled 100 units. The signal from the piezo was filtered, amplified and measured by a locally designed sample-and-hold circuit interfaced to a computer.

The acoustic stimulus was generated by a white-noise generator (MED associates, Fairfield, VT USA) and passed through a high frequency speaker located at the back of the Styrofoam chamber. The rise and fall were 5 ms and the white-noise burst lasted for 100ms. The startle data was collected via a custom written program on a Pentium 2 PC.

Acoustic startle chambers # 2: MED Boxes (Med associates, Fairfield, VT, USA)

A second set of acoustic startle chambers consisted of a rat holding cage (165mm x 80mm x 90mm) that was located within a sound-attenuating melamine chamber (600mm x 340mm x 560mm). Four of these chambers were located in a special sound attenuating room. Inside each chamber was a platform with a holding cage and a frame holding a light and a speaker. The floor, ceiling and walls of the rat holding cage were made of stainless steel horizontal bars, the floor bars were 0.45mm in diameter and spaced 15mm apart, while the walls and ceiling rods had a diameter of 0.25mm and were also spaced 15mm apart. Under each cage was a tray to collect excrement. The floor bars were connected to a constant current scrambled shock generator which delivered a computer controlled 600-μA foot-shock for 500ms. A maximum current of 600-μA current was used because, in shock sensitization research with humans, it has been reported to be the tolerable threshold (Greenwald, Bradley, Cuthbert, & Lang, 1998; Tursky, 1973) and is one of the most commonly used for eliciting a jump/flinch reaction in rats (Davis, 1986). Within the chamber a metal frame held a 2.8w lamp and a 60mm speaker that was 100mm away form the rat holding cage. The rat holding cage was mounted on a load cell-based startle platform (250mm x 115mm x 45mm). Movement amplitudes (500 mV peak voltage amplitude equals 100 units) were amplified, digitized and recorded by programmable Med associates software. This program could control the light, noise and foot-shock duration during all experimental phases. The acoustic stimulus was a 100-ms white noise burst with a rise and fall time of 10ms.

The holding cages and trays were cleaned using hot soapy water to remove contamination. The chambers' interior was lightly sprayed with a 70% alcohol solution to mask odour.

Paradigms: Shock Sensitization and Fear Potentiated Startle.

In this thesis two main paradigms were used, shock sensitization and fear potentiated startle. Both paradigms measure fear via augmented startle. Essentially, acoustic startle was measured before and after the manipulation, which was either presentation of a US or a CS. A significant difference between acoustic startle reflexes was a measure of fear.

Paradigm 1: Shock Sensitization (SS).

The shock sensitization paradigm was used for investigating the role of GABA, glutamate, dopamine receptors and protein synthesis inhibition in the basolateral and central amygdala, Experiments 1-5 and 13-17.

This paradigm is based on research conducted by Davis (1989). His main procedure involved delivering 40 noise bursts at 30 s ISI, 10 foot-shocks (0.6mA, of 500ms duration every second) followed by 40 noise bursts at 30 s ISI. The acoustic startle responses before and after footshock were compared. The difference between the means of these two groups of startle responses is a measure of fear. Davis' results showed robust shock augmented startle.

Three modifications were made to the original shock sensitization paradigm. The shock inter-trial interval was extended to 10 seconds, and the numbers of acoustic startle probes were reduced to 20 for each manipulation. An additional 20 probe trials were

presented before drug infusion. The collected data could then be compared to the 20 pre-shock trials in order to identify potential drug effects on startle. The 20 pre-shock trials were compared to 20 post-shock trials to study the drug effect on foot-shock-produced fear expression. To analyse drug effect on foot-shock reactivity, data were collected 250 ms before and during foot-shock.

Details of Shock Sensitization paradigm

Day 1: Rats were placed in the dark acoustic chambers and 5 minutes later were subjected to the baseline startle procedure. The baseline procedure involved 60 white noise presentations at 3 dB levels (91, 95, 99 dB) at 20 second intervals. The white noise presentations were in order of magnitude, and repeated 20 times, for a total of 60 presentations. Extreme noise levels (<110 dB) were avoided because they have been shown to independently produce sensitization (Plappert, Pilz, & Schnitzler, 1999). The startle data were used to establish an individual dB level that produced a mean score between 100 and 400 units for each rat (500 mV piezo displacement equals 100 units). The exposure to the white noise and chamber also allowed the rats to become familiar with the handling and testing procedure. Rats habituated with relative ease to noise bursts but continued to show startle (Chen, Ho, & Liang, 2000). If the rat showed no significant response to the highest dB noise bursts the auditory system may have become compromised during surgery and thus these rats were discarded from the study. After testing the rats were returned to their home cage.

Day 2: Using the startle data collected on day 1, the dB level that evoked an average score of between 100 and 400 units was programmed into the Med software and

used throughout the experiment. Figure 2.1 shows the flow diagram of the paradigm used on day 2.

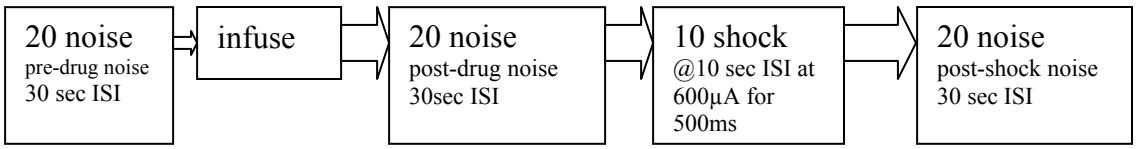

Figure 2.3: Flow diagram of the Shock Sensitization paradigm.

Each rat was placed in the rat retaining cage and exposed to 20 white noise bursts in the dark with a 30 s inter stimulus interval (ISI). It was then removed and infused with the appropriate drug for that experiment. (See infusion procedure). Subsequently the rat was returned to the retaining cage and after 5 minutes adaptation was exposed to a further 20 white-noise bursts at 30 s ISI. Ten foot-shocks of 600 μA (.6mA) at 0.5 seconds duration were delivered at 10 s intervals and this was followed by a further 20 white noise bursts at 30 s ISI. On termination of the experiment the rat was returned to its home cage. The 10 s interval between the shocks was brief enough to prevent the formation of a memory that could have affected later memory tests (Josselyn et al., 2001). The 600 μA (0.6mA) shock level induces tail flicking and vocalization and has been judged aversive (Illich, King, & Grau, 1995) and is comparable to that used by Davis(1989). The total duration of the experiment was no more then 26 minutes post infusion. It was therefore highly unlikely that, during such a short duration, the drugs would have fully dispersed to adjacent areas and thereby confounded the results (Westerink & de Vries, 2001).

Statistical Analyses and dependent measures

A repeated measures ANOVA was used to evaluate the significance of differences between the dependent variables. This was followed by simple effects analyses to determine the differences between group means. Repeated measures ANOVAs reduce the error variance between subjects and thus the number of animals needed. The statistical analysis package Statistica 6.1 from StatsSoft Inc., USA, was used for all analyses.

The dependent measures were 3 sets of acoustic startle data, pre-drug, post-drug and post-shock, and 2 sets of reactivity data collected before and during footshock.

Paradigm 2: Fear Potentiated Startle (FPS).

The second paradigm used was the fear-potentiated startle paradigm. This paradigm has been successfully developed to produce reliable fear conditioning that is stable, persistent and easy to measure (Davis, 1986; Falls, Miserendino, & Davis, 1992; Sananes & Davis, 1992). It was used to investigate the roles of GABA, glutamate and dopamine receptors and protein synthesis inhibition in fear expression to a CS in the basolateral and central amygdale (Experiments 6-12 and 18-23).Figure 2.2 illustrates the flow diagram of the fear-potentiated startle paradigm.

Day 1: Baseline.

During the baselining procedure all rats were exposed to 60 white noise bursts at three levels of intensity (91, 95, 99 dB) with a 20 second inter stimulus interval (isi). Each rat was allocated a dB level that produced an average startle response between 100 and 400 units (500mV displacement equals 100 units) and this was used throughout the procedure. Rats were returned to their home cage after the final white noise burst.

Day 2: Learning phase.

In order to ensure learning of the CS-US association conditioning sessions were conducted twice in one day. In both the am and pm sessions, rats were exposed to 15 light-shock pairings (3.5 s light followed by a 500ms shock, 600μA) with a randomly varied isi ranging from 56-180 s (Fendt, Koch, & Schnitzler, 1996; Sananes & Davis, 1992). The random presentation of the CS-US within the range of 56-180 seconds created unpredictability which has been shown to improve the rate of conditioning (Gallistel & Gibbon, 2000).

Day 3: Short fear test

The rats were exposed to a short fear test consisting of 10 noise trials followed by 5 noise and 5 light + noise trials. The inter-stimulus interval (isi) was 30 s for the 10 noise trials. The rats were exposed to 10 noise bursts to re-establish the baseline and reduce the variance between the responses. Following the 10 noise bursts, the rats received 5 white noise bursts (45 s isi) followed by 5 light + noise trials (isi of 45 s) during which the light was on for 3.5 s and then followed by a 100ms white noise burst. A significant increase between acoustic startle after CS presentation was an indication of fear.

Day 4: Test day.

AM 'reconditioning'

In the morning of the test day, rats were exposed to 5 conditioning trials with the same parameters as on day 2. This procedure allowed the rats to be trained further and also alleviated any potential extinction caused by the 5 light + noise trials from day 3. The procedure is similar to that adopted in research conducted by Fendt (2001) and

Schulz, Fendt, and Schnitzler (2002). However, these authors 'reconditioned' their animals 4 hours before testing, while the interval was 8 hours in the present study.

PM Testing

Each rat was tested individually and exposed to 20 white noise bursts (30 s isi), then infused (see infusion procedure) and returned to the startle chamber. Five minutes later it was presented with 20 noise bursts (30s isi) to test for a drug effect on the acoustic startle reflex. Thus a significant difference between pre-drug and post-drug may indicate a drug effect on startle expression. This was immediately followed by 10 white noise bursts (45 s isi) 10 light + white noise bursts (45 s isi) to test for any drug effect on fear expression. Fear expression was measured by subtracting data from the 10 light+ noise startle from those from the 10 noise startle trials. No significant difference indicated a drug effect.

The total testing duration under drug influence was no more than 30 minutes. The rats were returned to their home-cage after completion of the last light+noise trial.

Day 5: Final test, 24 h later.

During the early evening of the next day, the rats were tested without drugs to identify fear expression. They were placed in the startle chambers, adapted for 5 minutes and presented with 10 white noise (45 isi) bursts followed by 10 noise + 10 light (45 isi). A significant increase in the acoustic startle reflex after CS presentation was an indication of fear.

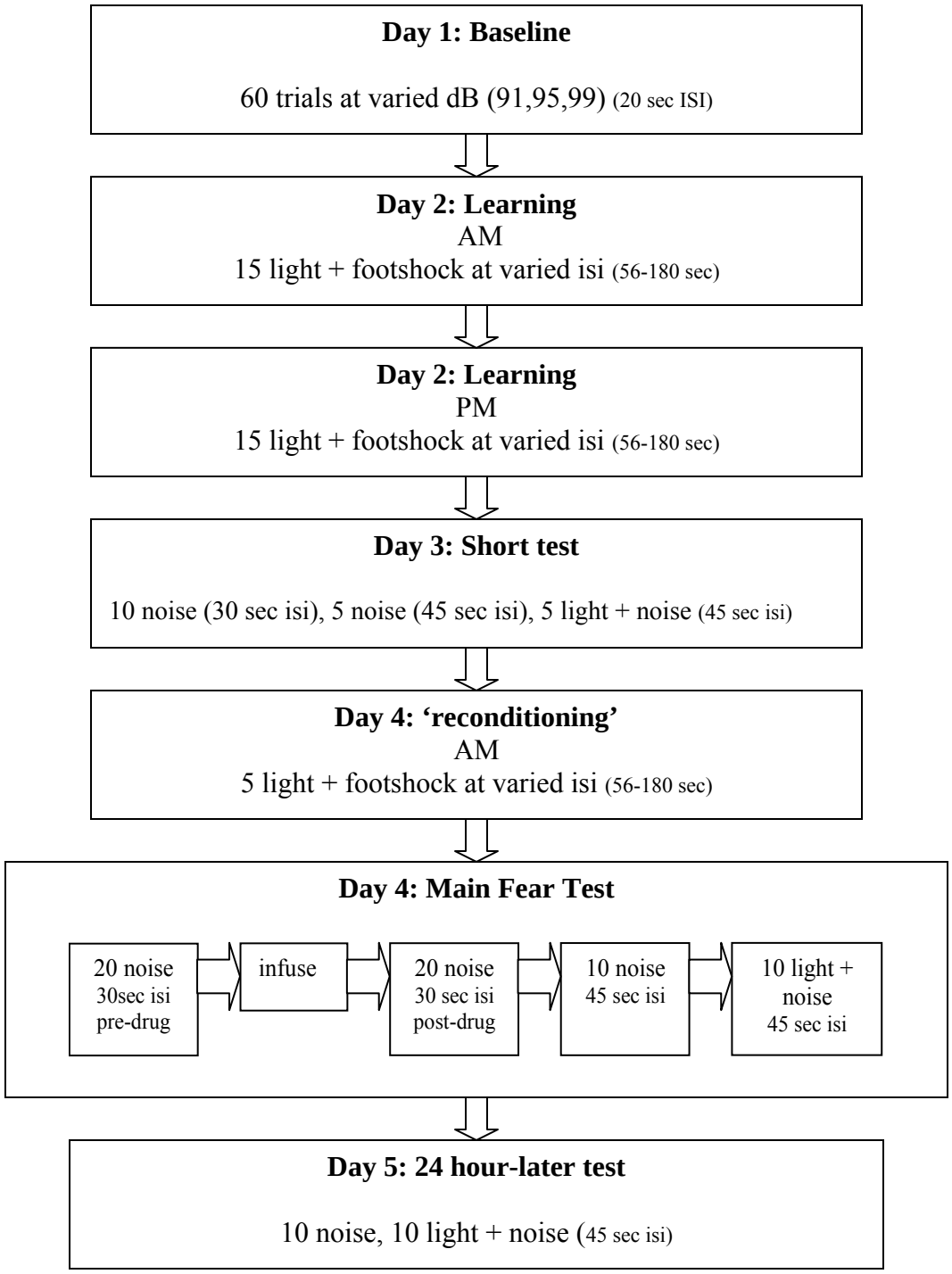

Figure 2.4: Flow diagram of fear-potentiated startle paradigm.

Statistical Analyses and dependent measures.

A repeated measures ANOVA was conducted for each of the four tests, namely the short fear test, pre-drug/post-drug, the main fear test and the 24 h later test. This was followed by simple effects analyses to determine the differences between group means.

Drugs, concentrations and drug formulae.

The drug concentrations (volume = 0.5 µl, flow rate = 0.5µl/ min) were based on previous research in which the drugs used in the present study were observed to have significant effects in the (BLA) and the central amygdala (CA)

An infusion rate of 0.5µl/ min is possibly the optimum rate for infusion across brain matter because it reduces leakage into adjacent areas, and backflow is kept to a minimum (Chen, Lonser, Morrison, Governale, & Oldfield, 1999). Relatively low drug concentrations and volumes were used because of the need to contain most of the active ingredients in the brain areas of interest. Specific research conducted by Westerink and de Vries (2001) showed that high concentrations (1-10 mmol millimole) are needed to reach substantial drug levels 1 mm away from infusion point. This is similar to the work of Edeline, Hars, Hennevin, and Cotillon, (2002), who noticed that with the smallest volume of 0.05µl at a concentration of 8.7mmol (1µg/µl), muscimol still diffused 2-3 mm from the injection site. The pharmacological action of lidocaine and muscimol lasts for at least 30 minutes (Martin, 1991).

In this thesis, the concentration ranged from 25nmol (nanomole) AP-5 to 0.00005nmol for the lowest muscimol concentration and experimental manipulations did not exceed 30 minutes. Because the spread of the drug was expected to be minimal and the manipulations were of a relatively short duration, it is reasonable to assume that the

drugs would not spread far and would not lose their potency. Since the basolateral and central amygdala are adjacent to each other, it is important to limit the diffusion of drugs and ideally, to prevent them from entering the intercalated cells. However, it cannot be assumed that some of the drugs did not enter these cells.

All drugs were dissolved in physiological saline (0.9% NaCl) which was also used as the control. All drug concentrations were in line with previous research either completed at this laboratory or at other institutes. References are given beside each drug.

Muscimol hydrobromide (muscimol) 0.5, 0.1, 0.01, 0.001, 0.0005, 0.0001 µg/µl (2.56 nmol-0.00005 nmol) used in the shock sensitization dose response curve in the basolateral amygdala (van Nobelen & Kokkinidis, 2006). For all other work a concentration of 0.005µg/µl (0.025 nmol) has been used (Laviolette & van der Kooy, 2001, 2004; Sanders & Shekhar, 1995). Empirical Formula (Hill Notation): $C_4H_6N_2O_2 \cdot HBr$, Formula Weight: 195.01.

DL-2-Amino-5-phosphonopentanoic acid (AP-5) 5.0, 2.5, 1.25, 0.6 and 0.3 µg/µl (25 nmol-1.5 nmol) was used in the shock sensitization dose response curve in the central amygdala. For all other works a concentration of 5.0 µg/µl (25 nmol) was used (Fanselow & Kim, 1994). Empirical Formula (Hill Notation): $C_5H_{12}NO_5P$, Formula Weight: 197.13.

6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX) 5.0 µg/µl (18 nmol) (Kim, Campeau, Falls, & Davis, 1993). Empirical Formula (Hill Notation): $C_9H_2N_4Na_2O_4$, Formula Weight: 276.12.

(±)-SKF-38393 hydrochloride (SKF 38393) 4.0 µg/µl (14 nmol) 2-4 µg/µl range successfully used by Nader and LeDoux (1999b); Zarrindast, Rezayof, Sahaei, Haeri-

Rohani and Rassouli, (2003). Empirical Formula (Hill Notation): $C_{16}H_{17}NO_2 \cdot HCl$, Formula Weight: 291.77.

(−)-Quinpirole hydrochloride (quinpirole) 3.0 µg /µl (12 nmol) (Gifkins, Greba, & Kokkinidis, 2002). Empirical Formula (Hill Notation): $C_{13}H_{21}N_3 \cdot HCl$, Formula Weight: 255.79.

Anisomycin from *Streptomyces griseolus* (anisomycin) 80.0 µg /µl (30 nmol) (Vianna, Szapiro, McGaugh, Medina, & Izquierdo, 2001). Empirical Formula (Hill Notation): $C_{14}H_{19}NO_4$, Formula Weight: 265.30.

RESULTS SECTION 1: Experiments 1-5.

Control Groups demonstrating the validity of the Shock Sensitization Paradigm

Fendt and Fanselow (1999) suggested that one of the leading questions concerning fear potentiated startle acquisition is the role of the unconditioned stimulus (US). For the conditioned stimulus (CS) and US to become associated a neural representation of the US must exist. Footshock has been suggested to elicit the central fear state necessary to facilitate the connection between the US and CS. However, it is difficult to directly examine the central fear state. One paradigm that is valid for examining a central fear state is the shock sensitization paradigm. It measures potentiated startle after rapid unpredicted footshock presentations and is a reliable index of fear in both humans (Grillon & Baas, 2003; Grillon, Baas, Lissek, Smith, & Milstein, 2004) and animals (Davis, 1989). Some critics however state that this paradigm represents rapid contextual conditioning (Richardson, 2000; Richardson & Elsayed, 1998). The following experiments show this is unlikely.

Experiment 1: Shock sensitization control group.

Davis (1989) reported that either 5 or 10 rapid foot-shocks doubled the mean startle amplitude in response to the noise stimulus. This increase in startle amplitude was judged to be a measure of a central fear state. To show that foot-shock enhanced startle, eight naïve rats were baselined on day 1, and on day 2 received 20 white noise bursts followed by 10 foot-shocks followed by 20 white noise bursts (see shock sensitization paradigm

for more detail). It was expected that after footshock application the startle amplitude would double. Figure 3.1 gives the mean startle amplitudes for these in the two blocks of 20 noise bursts. After exposure to foot-shock, startle amplitudes were significantly increased.

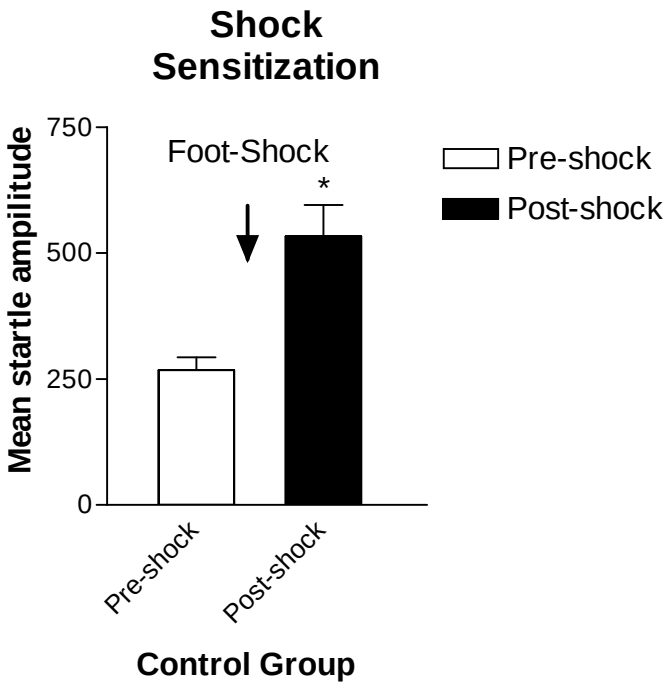

Figure 3.1: Shock sensitization.

Mean startle amplitude (±S.E.M) of rats before and after foot-shock application, * denotes significant difference between pre and post-shock, $p<0.05$.

A dependent t-test between pre-shock and post-shock revealed a significant positive effect of foot-shock on startle response, $t_7=-6.59$, $p<0.0003$, two tailed. In Figure 3.1 it is clear that rapid unpredictable footshock presentations doubled the acoustic startle response. Davis (1989) suggested that this increase was caused by the footshock, and the increase measured the internal fear state. However, it was possible that the increase in startle was due to a learned effect. To assess whether the rapid footshock presentations

resulted in the expression of conditioned fear as opposed to unconditioned fear, four further experiments were conducted namely, contextual conditioning, fear potentiated startle to an explicit cue, immediate footshock exposure and latent inhibition.

Experiment 2: Context conditioning.

Richardson (2000) suggested that the shock sensitization effect, shown above, could represent contextual conditioning (Richardson & Elsayed, 1998). Thus, during foot-shock exposure the animal learned that environmental cues could predict the onset of footshock. This learned behaviour was than expressed as an increase in startle after footshock presentation. Thus the increase in startle seen in Experiment 1 was not the result of footshock but an expression of fear to the context. To investigate this, 2 groups of 8 naïve rats each were base-lined with exposure to 60 noise trials at 20 s inter stimulus interval (isi) at 3 different intensities, 91, 95 and 99 dB. From this a dB level that reliably elicited startle responses between 100 and 400 units was selected for each animal and used throughout the experiment. Twenty four hours later, 1 group was exposed to 20 white noise bursts followed by 10 foot-shocks (600μA/0.5s) at 10 s ISI. The other group was exposed to noise only, and stayed in the chamber for the same duration of time. A day later both groups were exposed to 20 white noise bursts in the same chambers. Figure 3.2 illustrates the context conditioning paradigm. It was expected that if contextual conditioning took place then rats would show an increase in the startle response 24 h later. Thus comparing the startle data for Group 1 between day 2 and day 3 should show a significant increase, whereas for Group 2 no increase was expected.

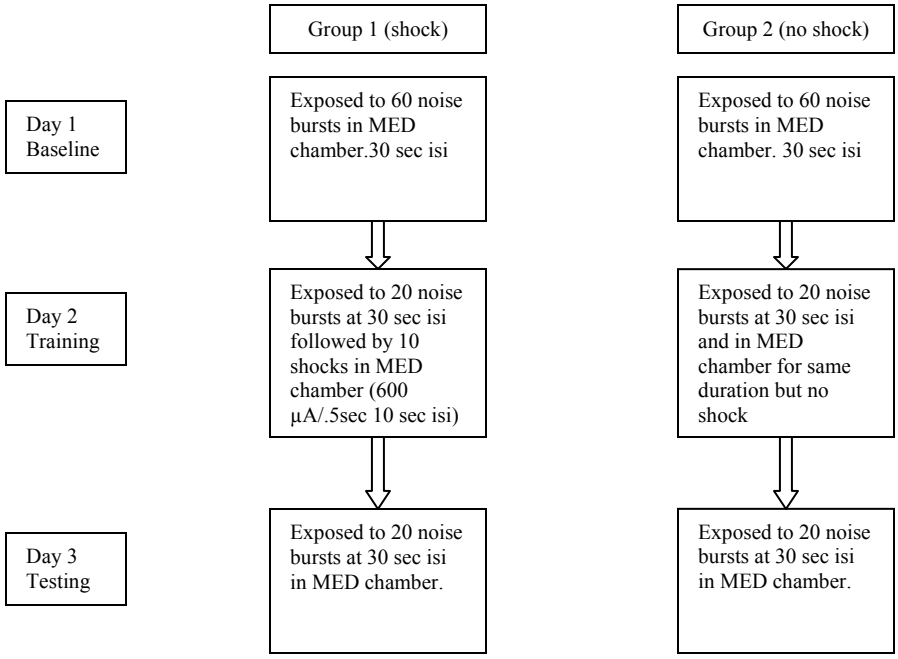

Figure 3.2: Paradigm for contextual learning.

The results, depicted in Figure 3.3 showed that both groups had comparable results, neither showed a doubling in startle amplitude on day 3 as compared to day 2.

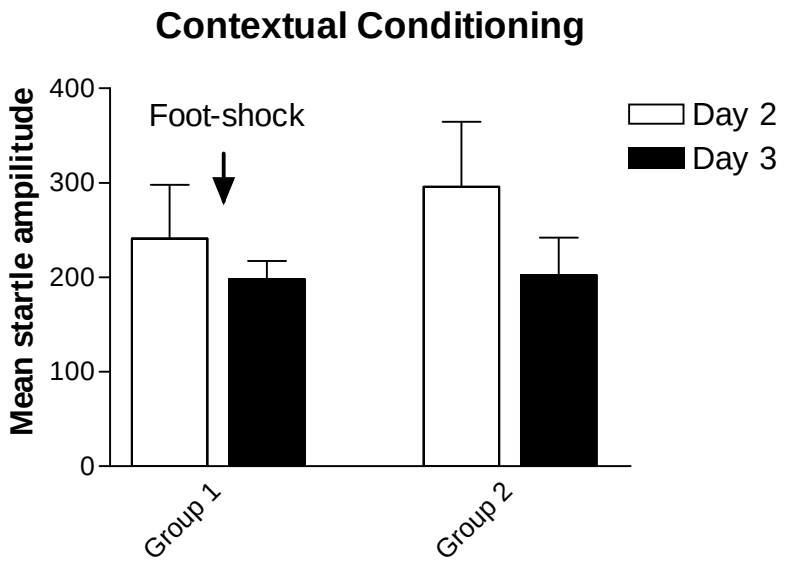

Figure 3.3: Contextual conditioning.

Mean startle amplitude (±S.E.M), neither Group1 nor Group 2 showed a significant difference between mean startle responses on day 2 (open bar) compared with day 3 (closed bar).

Figure 3.3 illustrates the mean (S.E.M.) startle data for Group 1 and 2, for days 2 and 3. T-tests for dependent means showed no significant differences between day 2 and 3, shocked group $t_7 = .80$, ns; non-shocked group $t_7 = .86$, ns.

These results showed that rapid footshock exposure failed to condition fear to contextual cues. No increase in startle was recorded 24 h later (van Nobelen and Kokkinidis 2006). One explanation was that the close successions of the shock trials were not conductive for learning the association between context and shock. For example, exposing rats to 3 foot-shocks with an isi of 3 seconds prevented the context-shock association from forming as measured by freezing to context 24 h later (Fanselow, DeCola, & Young, 1993). In the current experiment, rats also failed to show evidence of contextual conditioning.

Experiment 3: Pavlovian conditioning to rapid CS/US presentation.

One of the features of the shock sensitization paradigm shown in Experiment 1 is the close succession of the footshock. The rapid presentation may interfere with the formation of memories and the lack of specific cues in a contextual paradigm may have made it more difficult to learn the association between shock and context. However, perhaps presenting the footshock with an explicit cue such as a light could facilitate a learned response. Thus pairing light and shock in a temporal fashion may result in the formation of associations (Davis, 1986; Pavlov, 1932) that could be tested via an increase in startle response 24 hours later. Therefore, the possibility that learning may occur to rapid presentation of footshock paired with and an explicit cue, was also explored.

Figure 3.4 illustrates the simple paradigm in which 8 naïve rats were exposed to 60 base-line white noise bursts at intensities of 91, 95, 99 dB on day 1. From this a dB level that reliably elicited startle responses between 100 and 400 units was selected for each animal and used throughout the experiment. The rats were exposed to 10 light/shock (0.6mA/0.5s) pairings at 10 s ISI on day 2, and 10 noise, 10 light + noise exposures on day 3. If the light cue facilitated learning we would expect to see an increase in startle response after light presentation on day 3.

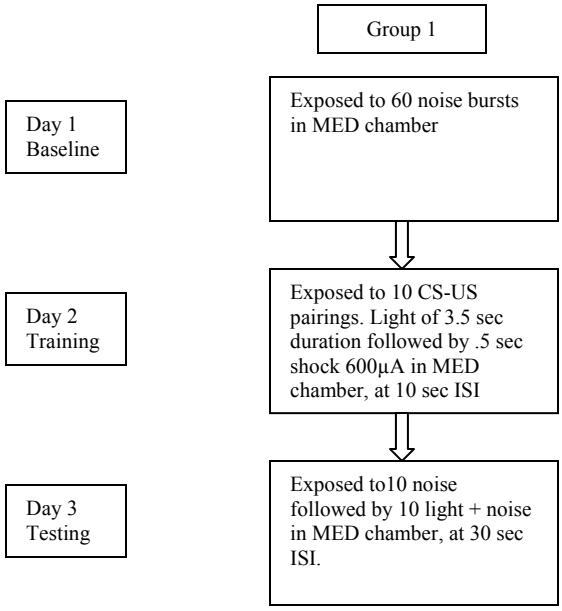

Figure 3.4: Paradigm for conditioning to an explicit cue.

Figure 3.5 shows the mean startle response to 20 noise and 20 + plus noise trials 24 hours after conditioning to rapid CS and US presentation. No significant difference was found between noise and light + noise mean startle amplitude.

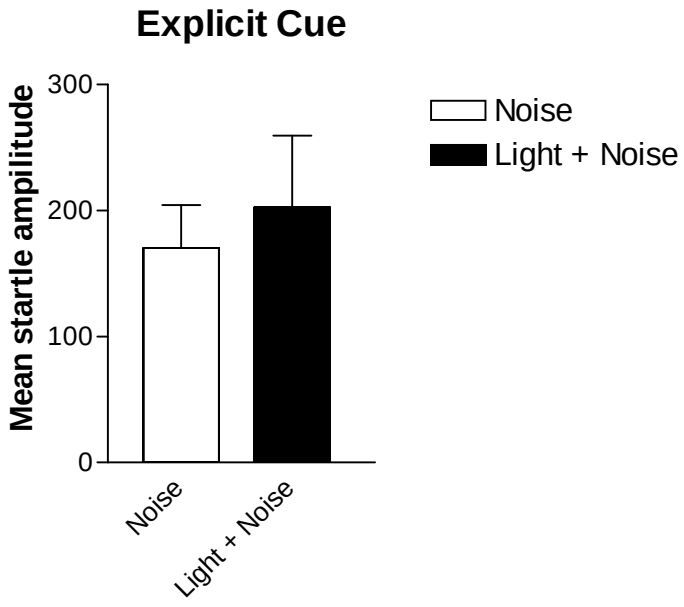

Figure 3.5: Explicit cue.

Mean startle amplitude (±S.E.M.) averaged across 10 noise and 10 light + noise trials, no significant differences were found.

A dependent t-test comparing the startle data between 10 noise and 10 light + noise on day 3 showed a non-significant result (t_7=.97, ns). In other words, as illustrated in Figure 3.5, when light and shock were paired and presented in close succession, conditioning to light did not occur (van Nobelen and Kokkinidis 2006). This suggests that rats were unable to learn fear even when the cue was obvious. In the shock sensitization paradigm in Experiment 1 no obvious cues were present during the rapid footshock presentation. In effect this means that even under less taxing conditions during Experiment 3 rats failed to show adequate fear conditioning. Thus it is unlikely that the enhanced acoustic startle effect shown in Experiment 1 was due to a learned effect.

Experiments 2 and 3 showed that the shock enhanced startle effect shown in Experiment 1 was not due to fear expression to either a contextual or an explicit cue.

Experiment 4: Immediate shock exposure.

The augmented startle after rapid footshock presentation as shown in Experiment 1 could still potentially be a learned effect, as the duration of the exposure to the experimental chambers prior to footshock may influence contextual learning (Lattel & Abel, 2001). Thus under-exposure to the context prevented the rats from observing the environment and thereby prevented the formation of a context-shock association during foot-shock presentation. Conversely, overexposure may weaken the contextual cues and thus also prevent associative learning. In Experiment 1 rats received exposure to the experimental conditions during the baselining procedure. Thus the duration of the 60 noise exposures to establish the baseline dB level may have been adequate enough for the rats to observe the contextual cues and this baselining procedure may have enhanced contextual conditioning. Therefore no pre-exposure to the testing chambers should result in poor contextual conditioning and subsequently no shock sensitization. To explore this option, rats were base-lined in PING chambers (see procedures and equipment on p 51 for detail). From this a dB level that reliably elicited startle responses between 100 and 400 units was selected for each animal and used throughout the experiment. The most obvious difference between MED and PING chambers were the floors and the walls. MED chambers have bars on the floor and walls while PING chambers have a flat plastic floor with wire mesh walls. These differences were important in creating different contexts, for rats have been shown to clearly discriminate between tactile cues and have good memory for them (Hughes, 2007).

Thus two groups of 8 naïve rats were exposed to 60 noise bursts in PING chambers on day 1 and on day 2 were placed into a MED chamber. One group received without

delay 10 footshocks (10 s isi) and this was followed by 20 noise bursts, and the other group just received 20 noise bursts. It was expected that due to the lack of exposure to the MED chambers, no shock sensitization would take place. The immediate shock paradigm is illustrated in Figure 3.6.

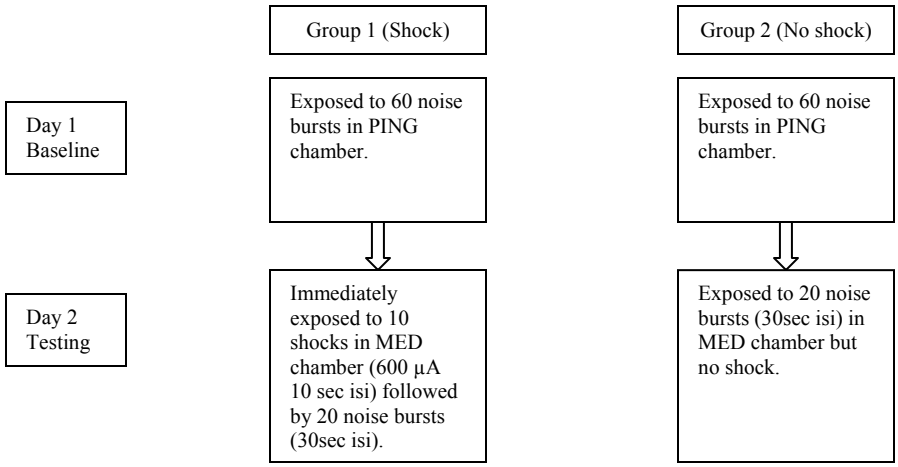

Figure 3.6: Paradigm for immediate footshock exposure.

Figure 3.7 illustrates the mean acoustic startle amplitude after immediate footshock and this is compared to mean acoustic startle amplitude after no footshock. The results showed a significant increase in startle after footshock.

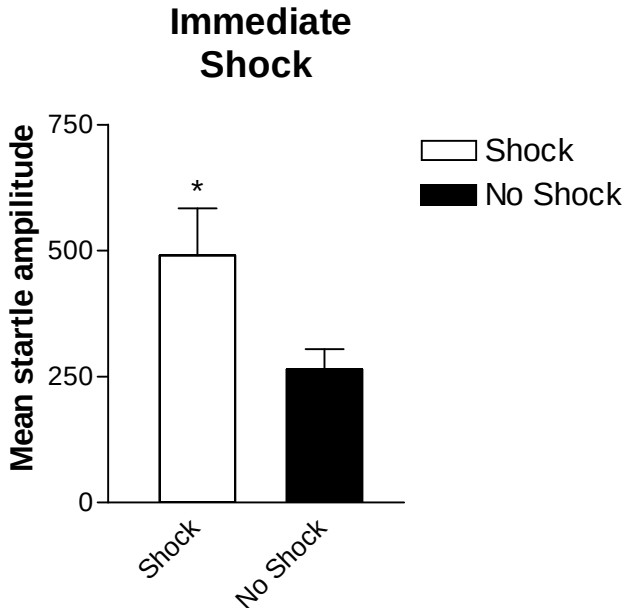

Figure 3.7: Immediate shock.

A significant difference between immediate foot-shock and no foot-shock as measured by mean startle amplitude (±S.E.M.), * denotes significant difference, $p<0.05$.

An independent t-test comparing the startle data from rats that received shock against those that did not, showed a significant difference ($t(14) = 2.22$, $p < .05$). Figure 3.7 shows that the rats that received foot-shock displayed significantly more startle than those that did not. This increase suggests that even without chamber pre-exposure, rats still showed robust shock sensitization, thus providing further evidence that shock sensitization is not an expression of contextual conditioning. This is in line with Davis (1989) who explored a variation to immediate foot-shock exposure. He suggested that potentially, dis-inhibition by foot-shock to noise bursts could be responsible for the increase in post-shock startle. Thus foot-shock reduces the habituation that occurs after repetitive stimulation with the same stimulus, noise. In his research rats were not subjected to any white noise prior to foot-shock but they still showed a sustainable

increase in startle after foot-shock compared to the non-shock group. These results provide evidence that neither exposure to the noise nor the chambers were critical in shock-augmented startle.

Experiment 5: Latent inhibition.

In contextual conditioning the environment is the conditioned stimulus (CS). Lubow and Moore (1959) suggested that non-reinforced exposure to the CS weakens the CS and makes it more difficult to later learn an association between the CS and US (Killcross, Kiernan, Dwyer, & Westbrook, 1998a, 1998b). Thus, if shock sensitization is contextual conditioning then pre-exposure to the MED chambers should weaken the potential CS and US association that Richardson (2000) suggests shock sensitization comprises. It would therefore be expected that pre-exposure will reduce it. To answer the question of whether pre-exposure to context reduced the shock sensitization effect, 2 groups of 8 naïve rats were baselined on day 1 to the PING chambers (60 white noise bursts). Again from this a dB level that reliably elicited startle responses between 100 and 400 units was selected for each animal and used throughout the experiment. On the following 3 days rats were placed for 20 minutes per day in the MED chambers, thereby allowing them ample opportunity to familiarize themselves with the chambers. This procedure was used by Kiernan and Westbrook (1993) and Killcross, Kieran, Dwyer and Westbrook (1998b) to demonstrate latent inhibition. On the 5th day one group was exposed to 10 foot-shocks (0.6mA/0.5s) at 10 s ISI followed by 20 white noise bursts at 30 s ISI. The other group received only the 20 white noise bursts at 30 s ISI. This paradigm is shown in a flow diagram in Figure 3.8.

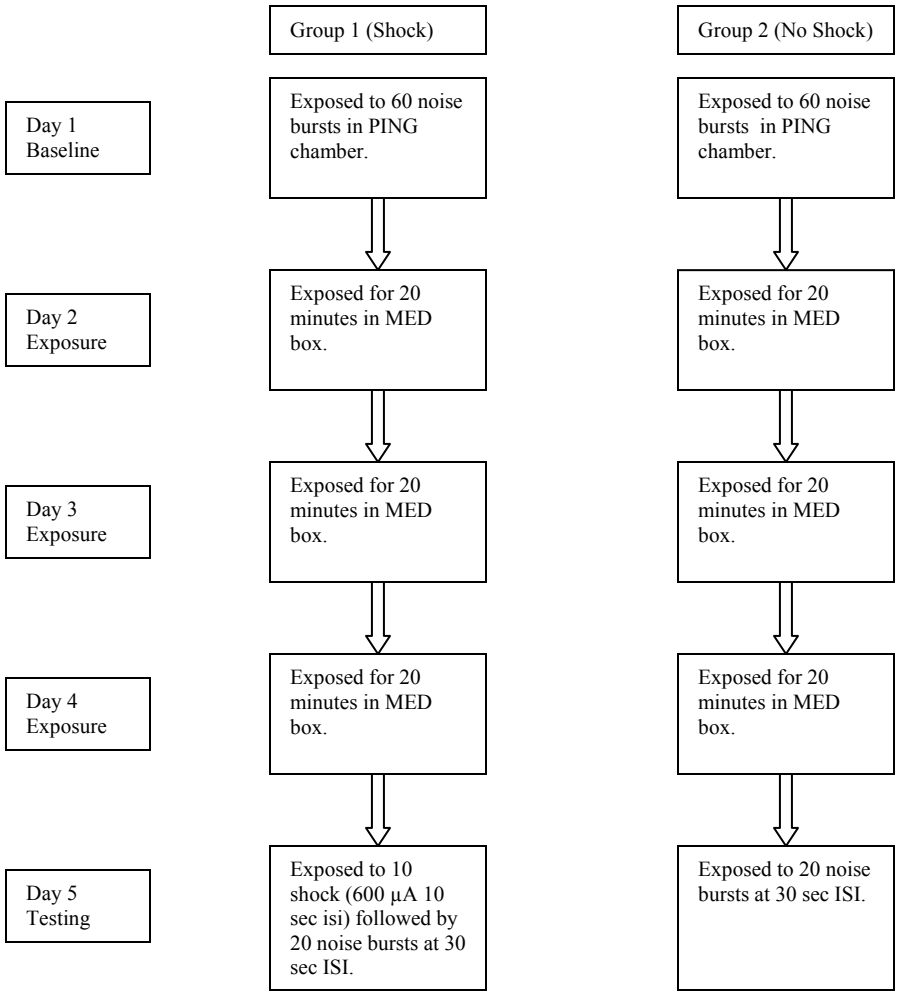

Figure 3.8: Paradigm for Latent inhibition.

Figure 3.9 shows the mean (±S.E.M) acoustic startle responses of the shocked Group and this were compared to the non-shocked Group. The results showed a significant increase in startle after footshock, suggesting the pre-exposure to the testing chambers had no effect.

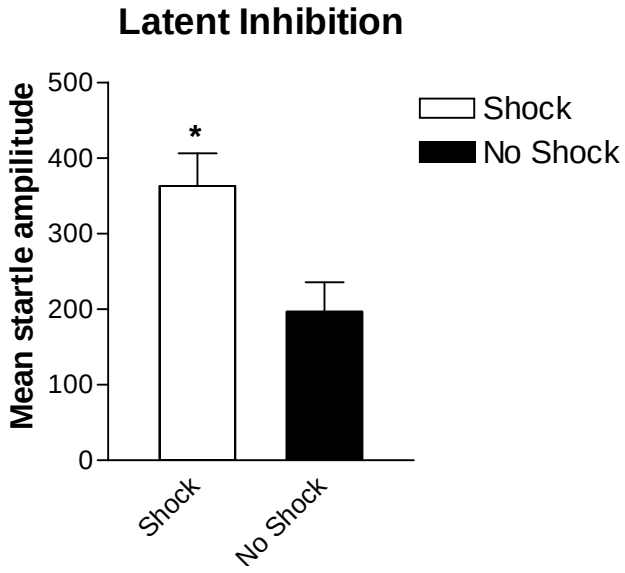

Figure 3.9: Latent inhibition.

A significant increase in mean startle amplitude (±S.E.M.) after footshock compared to no footshock, * denotes significant difference between shock and no shock group, $p<0.05$.

A t-test for independent means between the shock and non-shock groups showed a significant positive effect for shock ($t(14) = 2.82$, $p < .05$). Figure 3.9 shows rats that were shocked after 3 days of pre-exposure to the MED chambers still showed a significant increase in startle as compared to the group who did not receive footshock. The magnitude of the startle response in the shocked groups was nearly double as compared to the non-shocked group. This between-groups effect was similar to the within group effect seen in Experiment 1. These results imply that pre-exposure to context does not diminish the shock sensitization effect.

This is at variance with findings of Killcross et al. (1998b). They reported that pre-exposure significantly reduced freezing behaviour. But post-shock freezing behaviour may reflect a conditioned response to context (Fanselow, 1982; Kiernan & Cranney, 1992). Thus the freezing deficit noticed by Killcross et al. (1998b) would be expected,

since pre-exposure can reduce contextual conditioning if it is a learned response. Furthermore, when measuring freezing behaviour after foot-shock it was usual to see an immediate deficit (Rosen, 2004) which was not noticed when measuring startle (Davis, 1989). The above result that pre-exposure failed to reduce the shock sensitization effect suggests that the shock augmented startle was not a learned response. Therefore it is unlikely that the increase in acoustic startle produced by footshock as shown in Experiment 1 is a form of contextual conditioning.

Summary of results

All 4 Experiments (2-5) showed that the shock sensitization effect found in Experiment 1 was not influenced by external factors such as duration of exposure to the testing chamber, contextual and explicit cues. This suggests that no measurable conditioning had taken place and that the shock sensitization paradigm is a valid procedure to test for a central fear state. This means that the central fear state elicited by footshock can be measured as a difference between acoustic startle presentations before and after footshock.

Very little is known about the neural representation of the central fear state. What is known is that during fear acquisition, in the basolateral amygdala, the US and CS become associated (Miserendino, Sananes, Melia, & Davis, 1990; Romanski, Clugnet, Bordi, & LeDoux, 1993). Thus an obvious brain area to start investigating the central fear state produced by footshock was the basolateral amygdala. To this end rats were cannulised aiming for the basolateral amygdala and infused with either a $GABA_a$ agonist, two glutamate antagonists, a dopamine D1 or D2 agonist or a protein synthesis inhibitor, during the shock sensitization paradigm.

RESULTS SECTION 2: Experiments 6-10.

Analyses of footshock elicited fear arousal in the basolateral amygdala.

During the behavioural studies it was demonstrated that the shock sensitization paradigm is a valid measure for investigating a central fear state. It was shown that rapid footshock presentations did not produce a conditioned effect. That is, no measurable associations were formed. It also became clear that footshock produced a measurable fear state. A significant increase in startle responses after footshock as compared to before shock was an index of a central fear state (Davis, 1989).

Exposure to footshock elicits three reactions, a motor-reflex response, pain and a central fear state (Borszcz, 1993, 1995). It is most probable that this central fear state becomes associated with the CS during fear conditioning (Davis, 1989). Furthermore, it is likely that this same central fear state is expressed during CS presentation (Davis, 1986). This also suggests that the central fear state has a neural representation. Where the neural representation is stored and which neural system is responsible for execution has received little attention.

It has been clearly demonstrated that in the basolateral amygdala the CS / US associations are formed, stored and expressed (Anglada & Quirk, 2005; Davis, 2000; Helmstetter & Bellgowan, 1994; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Muller, Corodimas, Fridel, & LeDoux, 1997; Romanski, Clugnet, Bordi, & LeDoux, 1993; Schafe, Doyere, & LeDoux, 2005; Wilensky, Schafe, & LeDoux, 1999). This makes the basolateral amygdala a suitable location to examine the central fear state. This

was achieved by exposing rats to footshock during the shock sensitization paradigm. Rats with cannulae aimed at the basolateral amygdala were infused with one of several pharmacological agents. The $GABA_a$ agonist, muscimol, was infused to enhance the inhibitory action of the GABA receptor. Both the NMDA and non-NMDA antagonists, AP-5 and CNQX respectively, were used to inhibit the excitatory function of local glutamatergic receptors. Furthermore, to inhibit long term potentiation the protein synthesis inhibitor anisomycin was studied. Lastly, the effects of the dopamine agonists D1, SKF 38393 and the D2 agonist, Quinpirole were examined.

Experiment 6: The role of GABA during fear expression provoked by footshock.

The first drug whose effects were analysed was the $GABA_a$ agonist, muscimol. GABA is a principal inhibitory neurotransmitter in the central nervous system and found in abundance throughout the amygdala (Nicoll, Malenka, & Kauer, 1989). Benzodiazepines which comprise one group of minor tranquilizers, are reported to affect the $GABA_a$ receptor and have anxiolytic properties (Tallman & Gallager, 1985). The neuronal activity of the $GABA_a$ receptor is activated by muscimol and increases inhibitory functioning (Johnston, 1971). Muscimol is most often used in high doses as a local anaesthetic (Edeline, Hars, Hennevin, & Cotillon, 2002) and can prevent both acquisition and fear expression (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997). It is unclear if these failures are due to the inability to form an association between US and CS during conditioning and thus unable to learn the association, or if during fear expression, the rats cannot access the stored CS/US association.

An overlapping feature of both acquisition and fear expression is the ability to perceive and express a central fear state. It is possible that the inhibitory function of GABA neurotransmission prevented the expression of this central fear state. By infusing muscimol into the basolateral amygdala during the shock sensitization paradigm it was possible to see if the central fear state was affected. GABA neurotransmission does not appear to affect shock reactivity (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997) but it may affect the ability to produce a startle response.

To eliminate the possibility that startle expression was affected, rats were exposed to startle stimuli prior to drug infusion and the results were compared to startle under the drug's influence. It was expected that if muscimol modified the startle response, a significant difference between pre-drug/ post-drug startle would be found. To test for a change in the central fear state, post-drug/post-shock startle data were compared. It was anticipated that the infusion of muscimol could prevent the footshock-augmented startle, thus no significant differences between post-drug/post-shock were expected. A total of 56 rats were prepared and after histological verification two were eliminated from the data analyses.

To determine if anaesthetic levels of muscimol could suppress the shock-enhanced acoustic startle effect, rats were infused with a high dose of muscimol (0.5μg/μl). This effectively blocked the shock sensitization effect. To see if this anaesthetic effect was due to actual GABA neurotransmission, the dose was gradually lowered to 0.0001 μg/μl at which time the dose became ineffective and rats displayed robust shock sensitization. Figure 4.1 shows a dose response curve ranging from 0 (saline) to 0.5 μg muscimol.

Cannulae placements in the basolateral amygdala of the drug groups are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

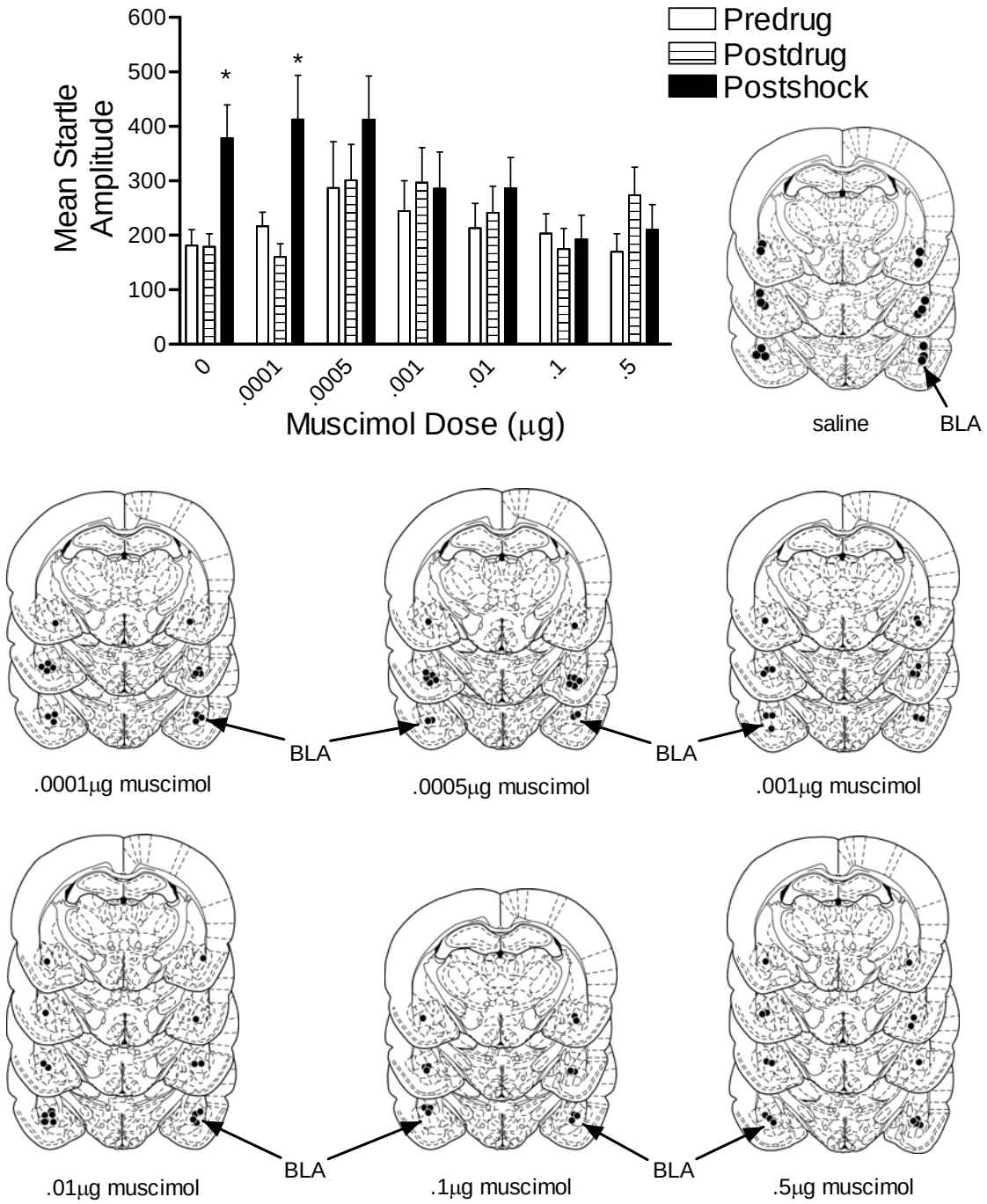

Figure 4.1: Muscimol dose response curve.

Mean (± S.E.M.) acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). Saline (N=8), 0.0001 Mus (N=8), Mus 0.0005 (N=8), 0.001 Mus (N=7), 0.01 Mus (N=8), 0.1 Mus (N=7), 0.5 Mus (N=8). * denotes a statistically significant increase between post-drug/post-shock responses for each drug, $P<0.05$. Cannulae

locations of the saline and muscimol groups depicted on schematics (Figs. 28-31) adapted from the Paxinos and Watson's (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 4.1 illustrates that the dose range from 0.001µg - 0.5µg muscimol reliably blocked the shock sensitization effect. A 7 (drug dose) x 3 (test) repeated measures ANOVA with the repetition on the test variable showed a drug x test interaction ($F_{12,94}$=2.01, P<0.04). Simple effects analyses showed that post-infusion startle amplitudes did not vary significantly from pre-infusion baseline startle in the saline (F= 0.001); 0.0001µg (F=1.05); 0.0005µg (F=0.69); 0.001µg (F=0.789); 0.01µg (F=0.26); and 0.1µg (F=0.30) muscimol groups. A non-significant elevation of post-infusion acoustic startle amplitudes over pre-infusion baseline levels was apparent in the 0.5µg muscimol group (F=3.5, P<0.07). This lack of effect of muscimol on startle suggests that the deficits shown were not due to suppression of startle. Foot-shock-enhanced startle responses were apparent in the saline group (F=11.2, P<0.002) and in the 0.0001 µg muscimol group (F=18.1, P<0.0001). This was also visible in the 0.0005 µg muscimol group but the difference between post-infusion and post-shock was not statistically significant (F=3.47, P<0.07). The magnitude of shock associated startle was similar to the saline and the 0.0001µg muscimol groups, raising the possibility of a ceiling effect.

The failure of footshock to produce enhanced post-shock startle was not due to the inability to process footshock. If muscimol affected the perception of footshock it was expected that the groups that showed no footshock-enhanced startle would also show diminished footshock reactivity.

Figure 4.2 shows the mean reactivity to foot-shock application 250 ms before and during testing. Rats in all groups clearly demonstrated robust reactivity to footshock.

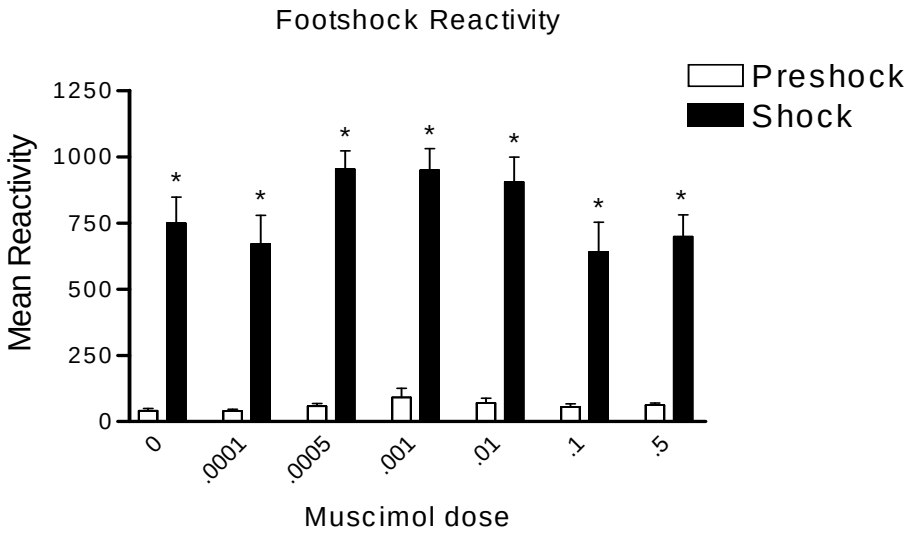

Figure 4.2: Shock reactivity muscimol dose response curve.

Mean reactivity (± SEM) of saline, and 6 different concentrations of muscimol, before and during foot-shock application. * denotes statistically significant increases between pre-shock and shock reactivity for each drug concentration, P<0.05.

A 7 (drug) x 2 (preshock-shock) repeated measures ANOVA with the repetition on the shock variable showed a significant effect for shock treatment ($F_{1,47}$=419.8, P<0.0001), and a near significant main effect for drug treatment ($F_{6,47}$=2.21, P<0.06) . It can be seen in Figure 4.2 that overall the responses of the 0.0005, 0.001 and the 0.01 µg muscimol groups were somewhat higher then the others.

The results of the dose-response curve illustrate that inhibitory GABA neurotransmission is involved in immediate fear expression as measured by the acoustic

startle paradigm. This claim is supported by Blair, Sotres-Bayon, Moita and LeDoux (2005) who suggested that muscimol could block the emotionally aversive qualities of noxious stimuli. The suppression of footshock-enhanced acoustic startle was not due to the rats' inability to react to the startle noise. All groups failed to show significant attenuation of startle between the pre-drug and post-drug test. In addition, shock perception was not attenuated. Thus muscimol did not affect the ability to startle or to detect the footshock but it did attenuate the expected increase in post-shock startle. As postulated by Borszcz (1993; Borszcz, 1995), the sensory characteristics of footshock consist of pain, spinal-reflexes and affective-motivational elements. According to this framework, muscimol did not affect the spinal motor reflexes because the rats showed strong reactivity to footshock during footshock presentation. Furthermore, startle expression itself was also unaffected. However, the expected startle increase caused by the immediate fear arousal was suppressed. Pain reduction can not be excluded but the basolateral amygdala does not appear to be involved in pain perception (Manning, Martin, & Meng, 2003; Neugebauer, Li, Bird, & Han, 2004), whereas the central amygdala is (Neugebauer, 2007; Neugebauer & Li, 2003; Neugebauer, Li, Bird, Bhave, & Gereau, 2003; Neugebauer, Li, Bird, & Han, 2004). It is possible that muscimol dissipated across the basolateral structure into the central amygdala, but as later experiments showed, small concentrations of muscimol do not suppress the immediate fear arousal caused by foot-shock in the central amygdala. Furthermore, it is unlikely that muscimol dissipated towards the perirhinal cortex because only high (0.5µg/µl) but not 'low' (0.25µg/µl) muscimol concentrations blocked the shock sensitization effect when applied to the perirhinal cortex (Schulz, Fendt, Richardson, & Schnitzler, 2004). Borszcz

(1993, 1995) thus suggested that shock may initiate a fear state. It is likely that additional activation of GABA neurotransmission by muscimol infusion prevented this. Likewise reductions of available intracellular GABA were found during and after fear conditioning (Stork, Ji, & Obata, 2002). The infusion of GABA antagonists produced anxiogenic effects (Sanders & Shekhar, 1995). Taken together, extra-cellular GABA appears to display homeostasis that alters after fear exposure.

These results may provide an explanation for results of Helmstetter & Bellgowan (1994) and Muller et al (1997) who inactivated the basolateral amygdala and prevented acquisition and expression of fear via GABAergic inhibition. Perhaps the rats were unable to experience fear during US presentation and this prevented the CS/US association. Moreover, rats were also unable to express fear during fear testing to a discrete cue. This suggests that GABAergic tone may affect fear expression and was accordingly evaluated by reducing the dose rate of muscimol during fear expression to CS presentation. The results are outlined in Section 3 Experiment 11.

The described results were interpreted as a GABAergic system being involved in immediate fear arousal produced by footshock.

Experiment 7: The role of glutamate receptor inhibition in fear expression provoked by footshock.

The basolateral amygdala is a site where associations between a CS and US are formed (Davis, 2000; Romanski, Clugnet, Bordi, & LeDoux, 1993; Schafe, Doyere, & LeDoux, 2005; Wilensky, Schafe, & LeDoux, 1999). The basolateral amygdala contains glutamate neurons, which are excitatory in nature (Vizi, 2003). These neurons are necessary in long-term potentiation and thus memory formation (Holscher, Gigg, &

O'Mara, 1999), but also in routine synaptic transmission (Bailey, Giustetto, Huang, Hawkins, & Kandel, 2000; Li, Stutzmann, & LeDoux, 1996; Weisskopf & LeDoux, 1999). Glutamate is implicated in the formation of the association between the CS and US (Goosens & Maren, 2004; Kim & Jung, 2006). For example, the infusion of the NMDAr antagonist AP-5 or the non-NMDAr antagonist CNQX prevented the formation of these associations (Campeau, Miserendino, & Davis, 1992; Maren, Aharonov, Stote, & Fanselow, 1996; McKernan & Shinnick-Gallagher, 1997). It was suggested that the formation of memory traces was inhibited.

Previous research has also shown that the glutamate antagonists AP-5 (Fendt, 2001; Lee & Kim, 1998; Lee, Choi, Brown, & Kim, 2001; Lindquist & Brown, 2004; Maren, Aharonov, Stote, & Fanselow, 1996) and CNQX (Kim, Campeau, Falls, & Davis, 1993) inhibited the expression of conditioned fear post infusion. Again an overlap exists between acquisition and expression, but is this a central fear state or a memory formation/expression deficit? Evidence suggests that NMDA prevents the formation of the CS-US association, but conflict exists over the role of NMDA in fear expression. Fendt (2001) proposed that NMDA receptors are important for synaptic transmission during fear expression. Perhaps the central fear state is guided by normal synaptic transmission as opposed to long-term potentiation induction. It is not yet clear if glutamate has a role in the expression of a central fear state produced by footshock.

To investigate this, 26 rats were surgically prepared and infused with saline, AP-5 or CNQX at dose rates that have been shown to prevent fear acquisition and expression (Kim, Campeau, Falls, & Davis, 1993; Roesler et al., 2000) The basolateral amygdala is sensitive to glutamate antagonists during fear expression. This is also demonstrated in

Experiments 12 and 13 whereby 5µg/µl AP-5 or 5µg/µl CNQX significantly reduced fear expression.

If the immediate fear effect produced by footshock has an associative element or relies on basal neural functioning then the glutamate antagonists should block the augmented acoustic startle. Alternatively, if the shock sensitization paradigm does not test for associations between context and US, but tests for unconditioned fear, it was expected that the infusion of glutamate would not prevent augmented acoustic startle responses.

Roesler et al. (2000) and Mesches, Bianchin and McGaugh (1996) showed that AP-5 and CNQX infusions did not inhibit footshock perception and thus it was expected that all groups would react vigorously to footshock stimulation. Of the 26 rats that were surgically prepared, 22 passed histological verification.

Figure 4.3 depicts mean startle amplitudes for the three treatment groups, saline, AP-5 and CNQX in the shock sensitization procedure. Cannulae placements in the amygdala of the drug groups are depicted on schematics adapted from the Paxinos and Watson's (1998) rat brain atlas, BLA, basolateral amygdala.

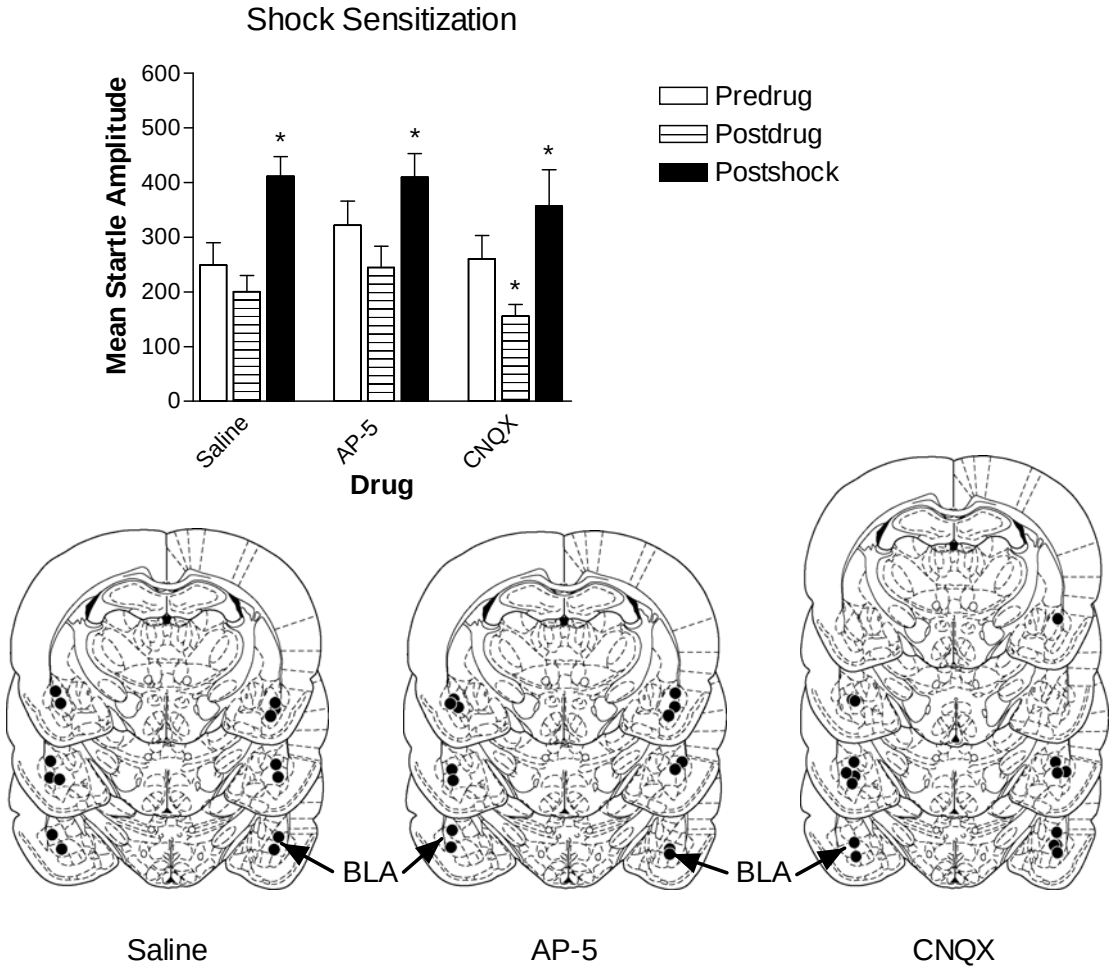

Figure 4.3: Effect of glutamate antagonists on shock sensitization.

Mean (± S.E.M.) acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). Rats infused with saline (N=7), AP-5 (N=7), and CNQX (N=8) showed statistically significant increases in post-shock startle relative to post-drug startle * = $P<0.05$. Additionally, CNQX infused rats showed a significant attenuation between pre-drug and post-drug, * = $P<0.05$. Cannulae placements for the three groups are depicted according to schematics adapted from Paxinos and Watson (1998); Figures 28-31 BLA, basolateral amygdala.

In Figure 4.3 it is evident that while under drug influence, all groups showed a significant enhanced acoustic startle response between post-drug infusion and post-shock. Additionally, CNQX infused rats displayed significantly attenuated acoustic responses

after drug infusion. A 3 (drug) x 3 (test) repeated measures ANOVA with the repetition on the test variables (predrug, postdrug and postshock) showed a significant main test effect ($F_{2,38}$=26.52, P<0.00001) and non-significant main drug effect ($F_{2,19}$=0.37 n.s.) and drug X test interaction ($F_{4,38}$=0.76 n.s.). The saline and AP-5 groups both showed a slight, but non-significant attenuation in startle amplitude after drug infusion. The CNQX group was affected, possibly because CNQX blocks the AMPA receptor responsible for basal neural functioning (Wang, Wilson, & Moore, 2001). This attenuation was not carried over during post-shock acoustic responses. After foot-shock application all groups showed significant increases in startle responses between post-drug and post-shock. Davis (1989) and the results of Experiment 1 showed that rats exposed to 10 rapid foot-shocks doubled their response to noise. This is similar to the saline and the two drug groups. All groups responded with comparable magnitudes in mean startle amplitudes during the post-shock test.

It could be argued that both AP-5 and CNQX were ineffective because of the concentration used. This is unlikely because the same concentration successfully blocked fear expression in the same area using a fear potentiated startle paradigm. This also indicates that glutamate neurotransmission is present in the basolateral amygdala but not required during the shock sensitization paradigm. Moreover both AP-5 and CNQX could successfully block the immediate fear arousal effect in the central amygdala showing that the shock sensitization paradigm is effective in other brain areas.

Since all groups reacted with vigour to noise after foot-shock application it is unlikely that these rats were unable to respond to footshock. To test this assumption, the data collected 250 ms before and during foot-shock application were analysed.

None of the drugs affected reactivity to foot-shock exposure. As depicted in Figure 4.4 all three groups showed significant reactivity to foot-shock. Pre-shock was the mean reactivity measured over 10 trials of 250ms before foot-shock onset. Shock was the average reactivity of 10 trials during foot-shock. A repeated measures ANOVA on the shock reactivity data revealed a main effect for shock ($F_{1,19}$=565.53,P<0.00001), demonstrating that the three groups of rats reacted significantly to foot shock presentation.

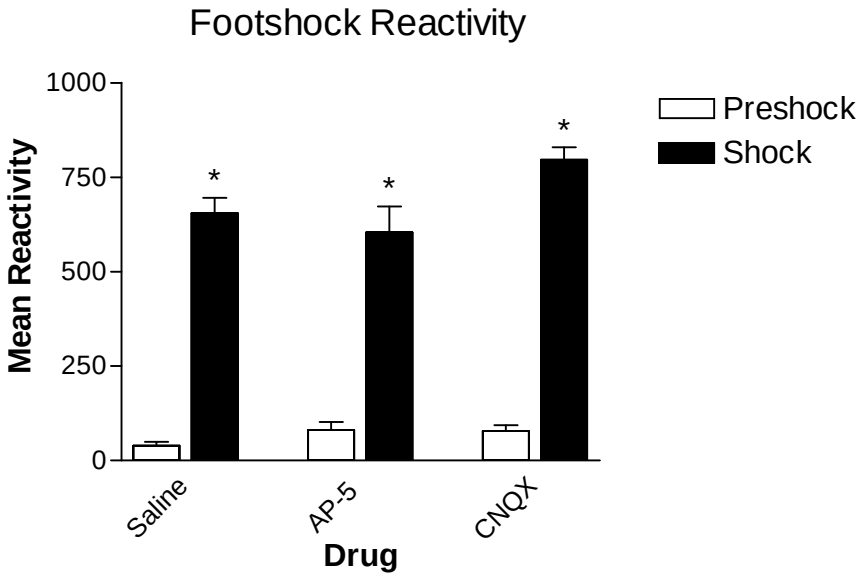

Figure 4.4: Effect of glutamate antagonists on shock reactivity.

Mean reactivity (± SEM) of saline, AP-5 and CNQX before and during foot-shock application.* denotes significant increases between pre-shock and shock within each drug, P<0.05

The non-significant effect of the high AP-5 (25 nmol = 5 µg/µl) dose on foot-shock confirmed results of Miserendino, Sananes, Melia and Davis (1990). They reported that the infusion of AP-5 produced no reduction in foot-shock reactivity during 10, 500ms of shock at .6 mA every 1 s. Furthermore, AP-5 does not affect flinch or jump thresholds

during inhibitory avoidance training (Roesler et al., 2000). This is consistent with the findings that AP-5 does not affect shock reactivity.

The above data suggest that inhibition of glutamate neurotransmission that previously had been shown to attenuate fear acquisition does not inhibit immediate fear arousal produced by footshock (i.e. in the shock sensitization paradigm). This implies that no measurable associative properties were evident. Moreover, immediate fear arousal is not mediated by normal glutamate regulated synaptic transmission, because immediate fear arousal was not suppressed. Furthermore, these drugs do not suppress the reaction to foot-shock. This brings some clarification to the relationship between glutamate, fear acquisition and expression of the CS-US association. Glutamate is not involved in the central fear state but may regulate other processes such as synaptic plasticity during the CS-US formation and control the expression of learned fear via routine synaptic transmission during fear recall (Fendt, 2001).

Experiment 8: The effect of anisomycin in fear expression provoked by footshock.

In the basolateral amygdala connections are formed between a CS and US, via long term potentiation. During the early phase, long-term potentiation does not immediately require protein synthesis to add extra AMPA receptors to the postsynaptic neuron (Lynch, 2004). However, during a later phase, minutes after long-term potentiation initiation, protein synthesis is a process that can modulate structural changes that are characteristics of this process (Abraham & Williams, 2003; Malenka & Bear, 2004). Moreover, fear memory consolidation is protein synthesis sensitive immediately after conditioning and this appears to involve NMDA receptor activation (Bourtchouladze et al., 1998). Thus, alterations of protein synthesis should not affect the shock sensitization

effect, because it does not appear to involve the formation of associations. The antibiotic anisomycin reduces, reversibly, up to 95% of the protein synthesis required in functioning cells (Grollman, 1967) and has been found effective in preventing long-term potentiation (Okulski, Hess, & Kaczmarek, 2002). To examine this, 10 rats were surgically prepared, but after histological verification 2 were excluded and not used in analyses. The anisomycin group was compared to the saline group from the glutamate study (Experiment 7). Figure 4.5 illustrates that the infusion of anisomycin indeed failed to affect the shock sensitization effect. Cannulae placements in the amygdala of the drug groups are depicted on schematics adapted from the Paxinos and Watson's (1998) rat brain atlas, BLA, basolateral amygdala.

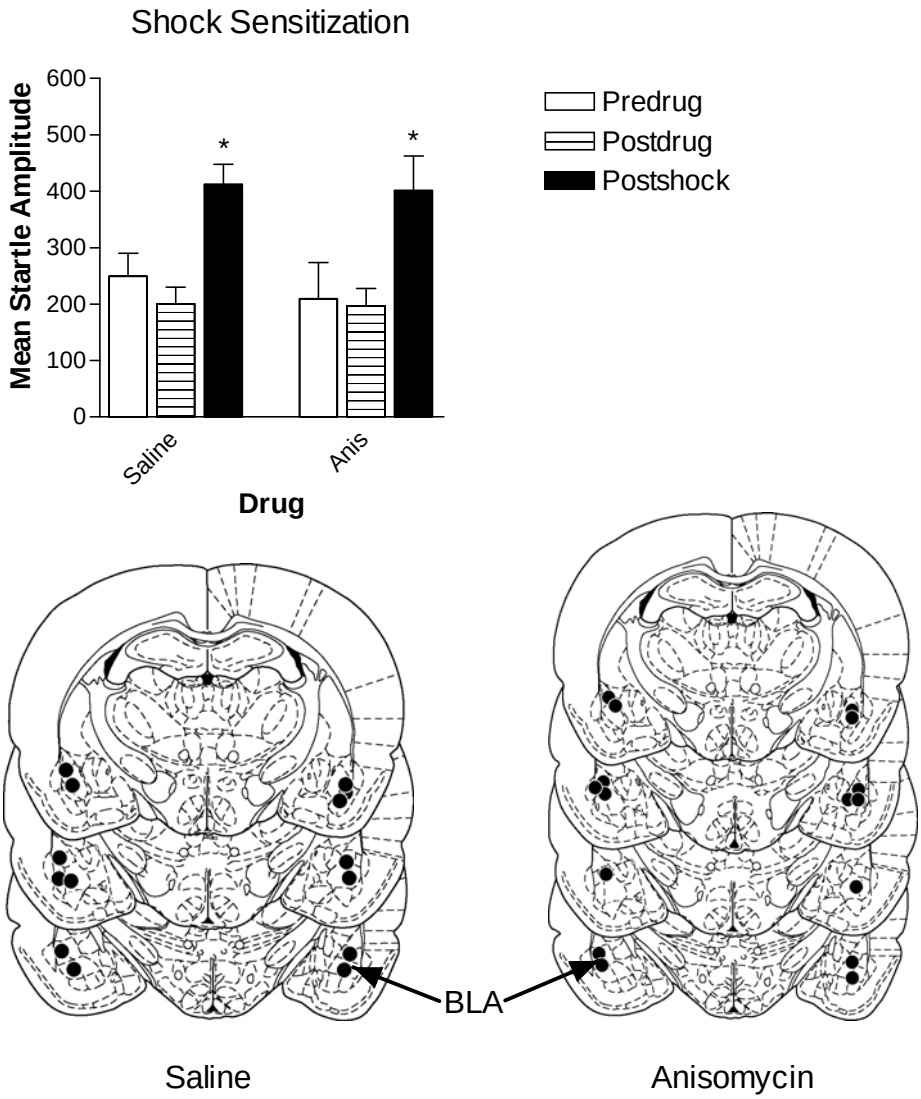

Figure 4.5: Effect of anisomycin on shock sensitization.

Mean (± S.E.M.) acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). Rats infused with saline (N=7) and anisomycin (N=8) showed significant increases in post-shock startle relative to post-drug startle, * $P<0.05$. Cannulae placements for the two groups are depicted according to schematics adapted from Paxinos and Watson (1998), Figures 28-31, BLA, basolateral amygdala.

As Figure 4.5 illustrates, both the saline and the anisomycin group showed significant increases between preshock and post-shock acoustic startle probes. A 2 X 2

repeated measures ANOVA showed a main test effect ($F_{2,26}$ =15.52, P< 0.00004). The failure of fear suppression was not due to inappropriate drug concentration. The same amount could significantly attenuate fear expression in Experiment 14 in Section 3. These results add to the knowledge that shock sensitization does not appear to test for fear/ context associations. Since neither AMPA nor NMDA receptors were involved in immediate fear arousal it was likely that protein synthesis inhibition would also fail to affect shock sensitization. In addition, anisomycin did not affect footshock perception. As Figure 4.6 illustrates, both groups showed significant reactivity during footshock, as compared to 250 ms before footshock.

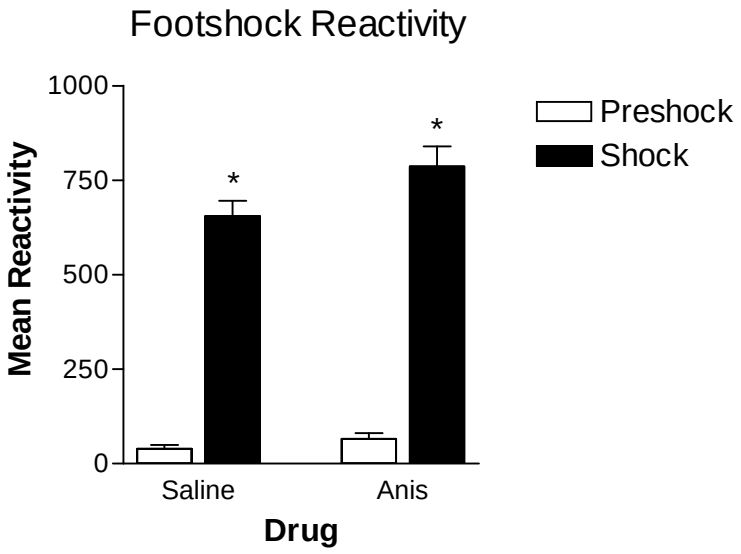

Figure 4.6: Effect of anisomycin on shock reactivity.

Mean reactivity (± SEM) of saline and anisomycin before and during foot-shock application.* denotes significant increases between pre-shock and shock reactivity within each drug, P<0.05

A repeated measures ANOVA performed on the shock reactivity data revealed a main effect for shock ($F_{1,13}$=461.59,P<0.00001), demonstrating that the two groups of rats reacted significantly to foot shock presentation.

Anisomycin has an important role in the structural changes of long term potentiation, and mRNA disruption prior to fear acquisition prevents associative learning (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999). The results presented here show that shock sensitization does not appear to involve measurable associations between context and footshock. This again suggests that immediate fear arousal produced by footshock does not involve rapid acquisition between context and shock but represents a central fear state.

Experiment 9: The effect of dopamine agonists in fear expression provoked by footshock.

The next group of experiments in this section investigated the role of dopamine agonists on immediate fear arousal produced by footshock. The basolateral amygdala contains both D1 and D2 receptor subfamilies and has been shown to have a strong inhibitory function in the lateral amygdala (Boysen, McGonigle, & Molinoff, 1986; Loretan, Bissiere, & Luthi, 2004). Dopamine has been shown to be involved in both fear acquisition and fear expression. But dopaminergic manipulations have exhibited inconsistent results during fear conditioning and fear expression. Notably, both the D1 (SCH 23390), (Greba & Kokkinidis, 2000) and the D2 (raclopride) receptor antagonists failed to suppress the central fear state produced during the shock sensitization paradigm, although higher >8μg but not lower doses of the D2 antagonist raclopride, have been shown to reduce the shock sensitization effect (Greba, Gifkins, & Kokkinidis, 2001). These same dopamine antagonists do reduce the expression of conditioned fear (Greba, Gifkins, & Kokkinidis, 2001; Waddington Lamont & Kokkinidis, 1998).

Furthermore, the D1 antagonist prevented the acquisition of second-order conditioning but the D1 agonist did not (Nader & LeDoux, 1999b). However, the D2 agonist, quinpirole, when injected systemically could block the acquisition of second-order conditioning via an impairment of recall (Nader & LeDoux, 1999a). Thus, both the D2 agonist and antagonist could block fear expression. Further evidence suggests that activation of dopamine receptors decreases neuronal firing and thus attenuates excitatory outputs of the basolateral amygdala (Loretan, Bissiere, & Luthi, 2004; Rosenkranz & Grace, 1999). This mechanism may decrease the central fear state. Although it is likely that dopamine may also affect memory retrieval, it cannot be ruled out that part of the memory retrieval involves activation of the central fear state. The role of dopamine agonists on a central fear state have not been studied directly. To investigate this, 22 rats were surgically prepared and infused with either the dopamine D1 agonist SKF 38393, or the D2 agonist quinpirole, and manipulated in the shock sensitization paradigm. After histological verification 6 rats were excluded because of inappropriate cannulae placements. The results were compared to the saline group from Experiment 7.

Figure 4.7 shows the mean startle amplitudes pre and post drug infusion and after foot-shock administration. Rats were infused bilaterally into the basolateral amygdala with 4.0 μg/μl SKF 38393 (N=8) or 3.0μg/μl quinpirole (N=8) and compared to the saline group from experiment 7. Cannulae placements in the amygdala of the drug groups are depicted on schematics adapted from the Paxinos and Watson's (1998) rat brain atlas, BLA, basolateral amygdala.

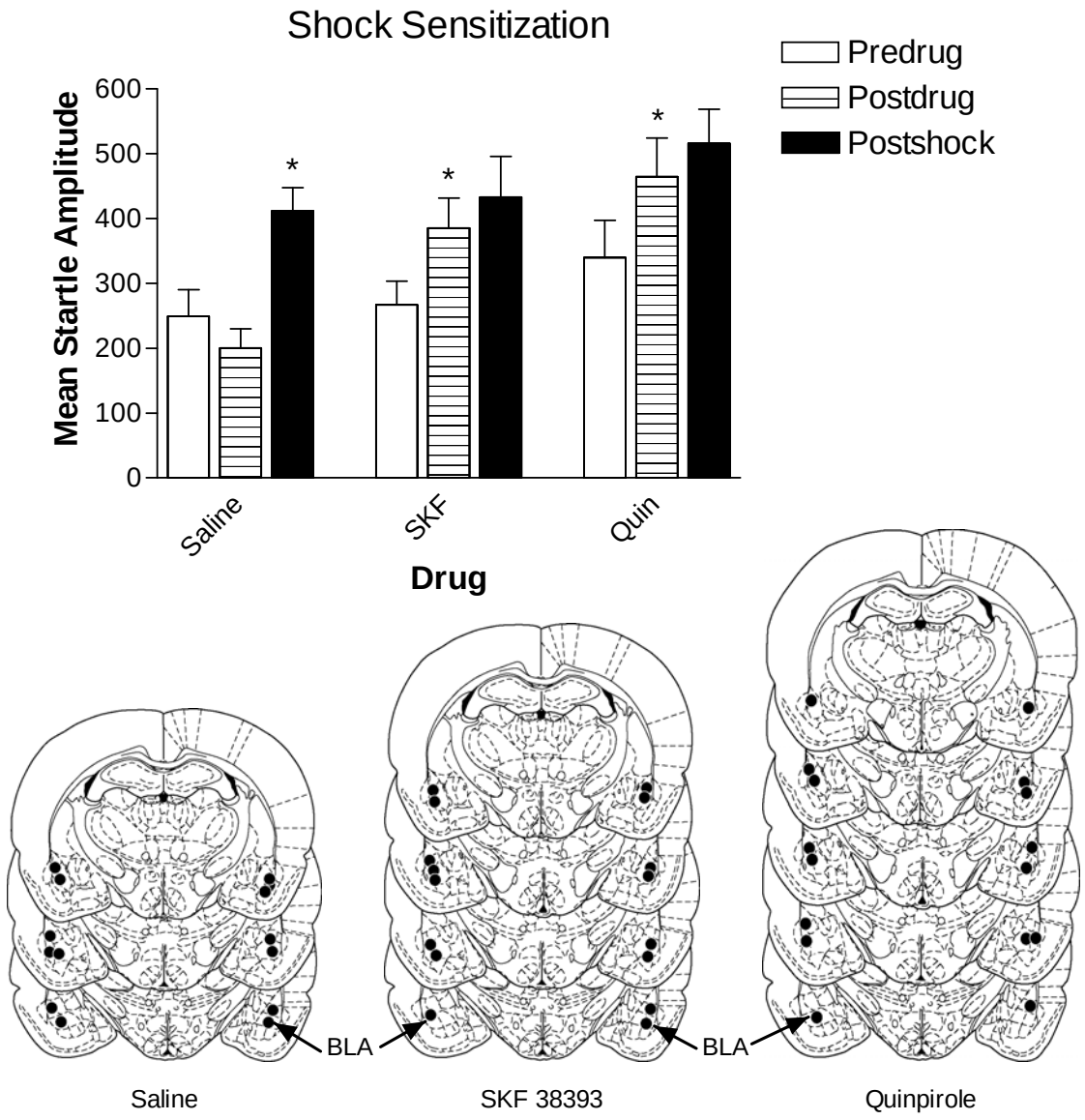

Figure 4.7: Effect of dopamine D1 and D2 agonists on shock sensitization.

Mean (± S.E.M.) Acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). * denotes significant within drug differences between pre-drug-post-drug (SKF 38393 and quinpirole) or post-drug-post-shock (saline), $P<0.05$. Cannulae placements for the three groups are depicted according to schematics adapted from Paxinos and Watson (1998), Figures 28-32 BLA, basolateral amygdala.

Figure 4.7 illustrates that both the SKF 38393 and quinpirole group showed a significant increase in startle amplitude between pre-drug startle and post-drug infusion startle but no further significant augmentation was evident after foot-shock administration. These groups showed post-shock means similar to the saline group, suggesting a ceiling effect, because the post-shock reactivity was augmented, albeit not to a statistically significant level. Moreover, if the D1 or D2 agonist affected fear arousal some startle attenuation would be expected, but on the contrary an augmentation of post-shock startle was evident. Therefore, it is unlikely that D1 or D2 agonists affected the central fear state produced by footshock. Additionally, these drug concentrations were effective in suppressing fear expression during fear-potentiated startle when infused into the basolateral amygdala, see Experiments 15 and 16. Thereby suggesting that D1 and D2 receptors are available in the basolateral amygdala but these are not used for the expression of a central fear state.

A repeated measures ANOVA 3 (drug) X 3 (test) with the repetition on the test variable showed two main effects, namely a drug main effect ($F_{2,20}$=3.54, P<0.05), and a test main effect ($F_{2,540}$=17.46, P<0.000001). Because the interaction approached significance ($F_{4,40}$=2.37, P= 0.07), simple effects analyses were performed and revealed that the saline group showed a non-significant effect between pre-drug and post-drug (F=0.73, P=0.40) but a significant effect between post-drug and post-shock (F=26.32, P<0.0001). Simple effects analyses also showed that both the SKF 38393 group (F=4.89, P=0.04) and the quinpirole group (F=5.48, P=0.03) exhibited significant increases in acoustic startle responses after drug infusion.

These increases suggested an augmentation of general movement, either caused by the dopamine agonists or as an expression of agitation from the infusion manipulation. This movement was marginally increased after footshock presentation. However, no significant differences were found between post-drug and post-shock startle amplitudes, SKF 38393 ($F=1.56$, $P=0.23$), quinpirole ($F=1.77$, $P=0.20$). The infusion of dopamine agonists may enhance overall motor activity, but this was not reflected in significant post-shock scores. Moreover, a similar pre-drug post-drug comparison was conducted in Section 3, Experiments 15 and 16, and it was reported there that neither SKF 38393 nor quinpirole significantly increased post-drug startle responding. However, the pre-drug/post-drug test was part of a slightly different paradigm. Furthermore, systemic injections of either SKF 38393 (Borowski & Kokkinidis, 1998) or quinpirole have no measurable effect on acoustic startle. Thus, the above unexpected increase in post-drug startle was inconsistent with other research. It was therefore concluded that a ceiling effect had operated because the magnitude of shock-associated startle was similar to that of the saline group. This was the only group that showed significant shock-augmented startle.

To further investigate the significant increase in post-drug acoustic startle, the footshock reactivity during footshock was analysed. Reactivity was measured 250 ms before and during foot-shock administration. In Figure 4.8 it is clear that all 3 groups show similar foot-shock reactivity but, more importantly, during the period before footshock both dopamine groups showed no significant agitation as compared to saline controls. Thus the rats under influence of dopamine agonists did not behave significantly differently from the saline control group.

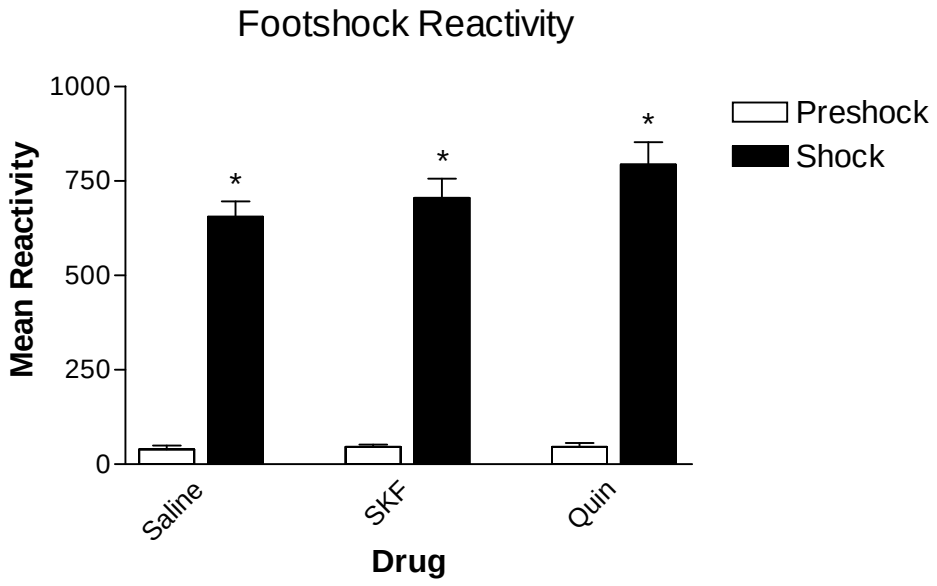

Figure 4.8: Effect of dopamine D1 and D2 agonists on shock reactivity.

Mean reactivity (± SEM) of saline, SKF 38393 and quinpirole before and during foot-shock application.* denotes significant increases between pre-shock and shock reactivity for each drug, $P<0.05$.

To evaluate if all groups could detect the foot-shock, a repeated measures ANOVA (3 drug X 2 preshock vs. shock) was performed. This revealed a robust main effect for shock ($F_{1,20}$ =468.80, $P< 0.00001$). Foot-shock significantly increased movement amplitudes and was unaffected by drug infusion. As can be seen in Figure 4.8, all groups reacted in a comparable manner to footshock.

Overall, dopamine agonists do not appear to affect immediate fear arousal caused by rapid footshock presentation thereby suggesting that dopamine receptors are not involved in the processing of the central fear state during this paradigm. However, under a different paradigm, activation of the D1 and D2 receptors, via intraperitoneal injections, heightened fear responses. These discrepancies have been attributed to differences between aversive paradigms (Reis, Masson, De Oliveira, & Brandao, 2004). Whereas,

chronic cocaine exposure has been shown to enhance available dopamine concentrations (Pettit, Pan, Parsons, & Justice, 1990), these increases do not affect shock sensitization (Willick & Kokkinidis, 1995). Thus the role of dopamine is at least ambiguous and may depend upon the type of fear examined.

Experiment 10: The effect of a low dose of muscimol in fear expression provoked by footshock.

The last experiment conducted in this Section was the infusion of a low dose of muscimol during the shock sensitization paradigm. Because part of this research concerned the specific role of GABA neurotransmission in both the basolateral and central amygdala, it was necessary to establish a drug concentration that would not act as a local anaesthetic but could still inhibit neural processes. Thus a low concentration of 0.005 μg/μl was chosen because it is in line with previous research conducted by Laviolette and van der Kooy (2001; 2004) who reliably conditioned rats to show place preference after muscimol infusion into the ventral tegmental area. Moreover, this dose rate was 5 times the lowest effective concentration (0.001μg/μl) reported in the dose response curve in Experiment 6. It was expected to be an effective dose rate but without the potentially anaesthetic properties found in higher concentrations.

Thus, 12 additional rats were surgically prepared, infused with 0.005 μg/μl muscimol and exposed to the shock sensitization paradigm. After histological verification 4 rats were excluded from the analyses. The muscimol group was compared to the saline group from Experiment 7. The results are illustrated in Figure 4.9 where it is demonstrated that 0.005 μg/μl muscimol also blocked the immediate fear arousal produced by foot-shock. Cannulae placements in the amygdala of the drug groups are

depicted on schematics adapted from the Paxinos and Watson's (1998) rat brain atlas, BLA, basolateral amygdala.

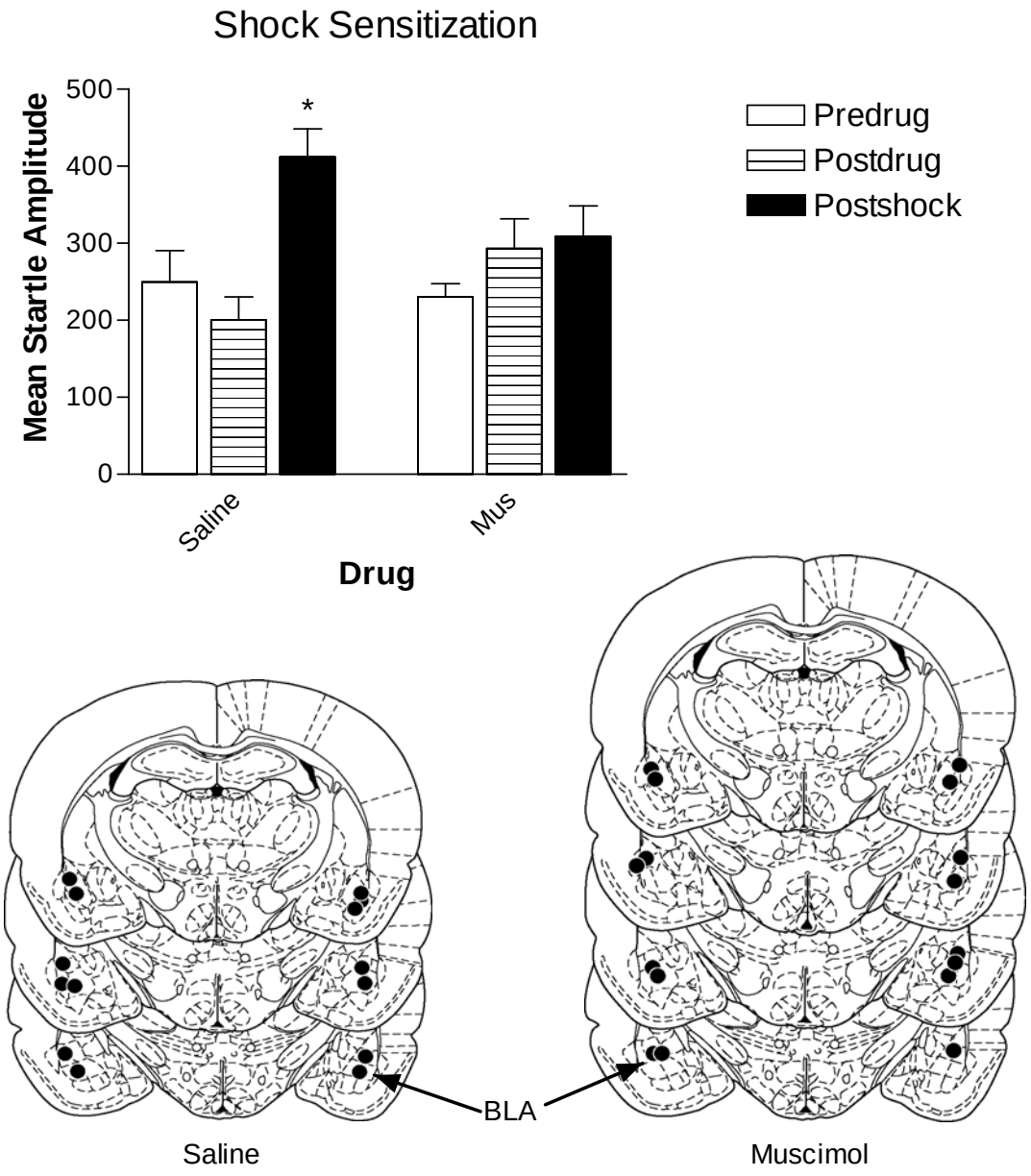

Figure 4.9: Effect of 0.005µg/µl muscimol on shock sensitization.

Mean (± S.E.M.) Acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). * denotes a significant increase between post-drug/post-shock in the saline group, $P<0.05$. Cannulae placements for the two groups are depicted according to schematics adapted from Paxinos and Watson (1998), Figures 28-31. BLA, basolateral amygdala.

Figure 4.9 clearly demonstrates that the low dose-rate of 0.005 µg/µl muscimol attenuated immediate fear arousal by foot-shock. A 2 X 2 repeated measures ANOVA showed a significant interaction ($F_{2,26}$ =5.70, P< 0.009) and a significant main test effect ($F_{2,26}$ =10.89, P< 0.0004). Simple effects analyses showed no significant differences between pre-drug and post-drug for both the saline and muscimol group (F=2.05, P=0.18; F=3.91, P=0.07) respectively. Conversely, a significant difference was noted between the saline post-drug-post-shock test. Rats showed a significant increase in startle responses after footshock (F=26.22, P<0.0002). The muscimol infused rats showed no notable increase after footshock, F=0.17, P=0.69).

This lack of a shock sensitization effect was not due to the rats failing to respond to footshock. Figure 4.10 shows the footshock reactivity measured 250 ms before footshock (pre-shock) and 250 ms during footshock (shock) presentation.

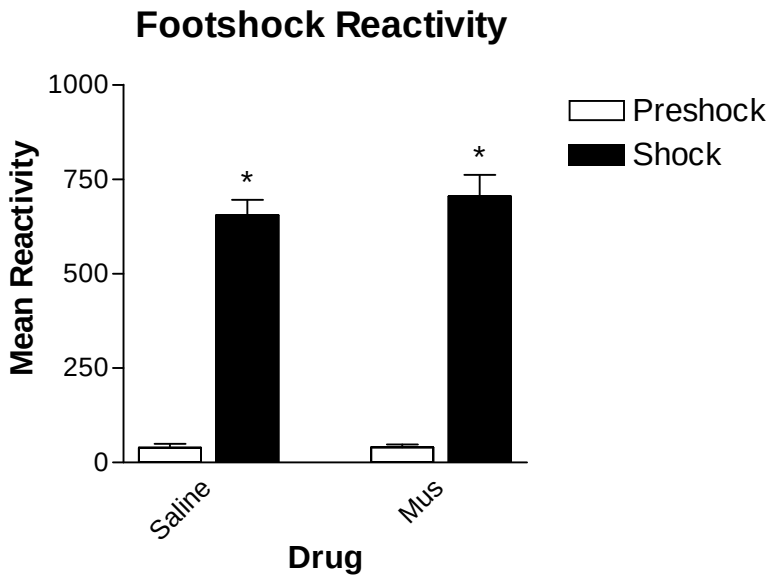

Figure 4.10: Effect of 0.005µg/µl muscimol on shock reactivity.

Mean reactivity (± SEM) of saline and muscimol, recorded 250 ms before and during foot-shock application.* denotes significant increases between pre-shock and shock within each drug, P<0.05.

Figure 4.10 shows that footshock reactivity was not affected by the infusion of muscimol. Statistical analyses using a 2 X 2 repeated measures ANOVA showed a main shock effect ($F_{1,13}$ =348.35, P< 0.0000).

Summary of results

This section investigated neural systems in the basolateral amygdala responsible for fear expression provoked by foot-shock. In Experiments 6 and 10 it was clear that activation of the GABAergic system controlled the expression of immediate fear arousal produced by footshock. Thus the basolateral amygdala is at least one of the areas where a neural representation of a central fear state can be located. As Experiment 6 demonstrated, inhibition of GABA neurotransmission with a low dose of muscimol prevented the central fear state/ affective motivational component but not the physical expression of the spinal reflex during the foot-shock application. In addition, the low dose of muscimol was unlikely to have any anaesthetic effect because it will not prevent learning of fear-potentiated startle. A minimum dose of 0.1 μg/μl muscimol is required for this (van Nobelen & Kokkinidis, 2006).

Experiment 7 showed that glutamate inhibition failed to affect fear expression produced by footshock. This also implies that with this paradigm, no specific testable associations between context and shock were formed because fear was freely expressed during the final acoustic startle probes.

Experiment 8 analysed the effect of anisomycin on immediate fear arousal and, as predicted, failed to suppress the fear arousal effect.

Experiment 9 showed that the dopamine D1 and D2 agonists did not prevent the expression of fear produced by footshock. Furthermore, the dopamine agonists did not affect footshock reactivity.

Experiment 10 showed that a small concentration of muscimol could significantly reduce shock augmented startle.

To investigate if the fear expressed during footshock presentation has the same neural underpinnings as fear expressed to CS presentation, rats were surgically prepared and trained in a classical fear potentiation paradigm to express fear to a CS. Prior to CS presentation rats were infused with drugs that affected either the GABAergic, glutamatergic, or dopaminergic systems or with a de novo protein synthesis inhibitor. It was expected that if the central fear state provoked by footshock is the same as that elicited by the CS, then the low dose of muscimol would suppress fear expression to CS presentation. These results are presented in Section 3.

RESULTS SECTION 3: Experiments 11-16.

The role of GABA, glutamate, dopamine, and protein synthesis in the expression of fear potentiated startle in the Baso-lateral amygdala.

During classical fear conditioning the CS and US are paired in a temporal pattern whereby the US either co-terminates with the CS or is presented immediately after cessation of the CS. These pairings produce reliable, measurable responses upon later presentation of the CS (Davis, 2000; Fendt & Fanselow, 1999). The US used in fear conditioning is aversive, and usually consists of footshock. Footshock has separate but interconnected qualities; consisting of reflexes, pain perception and an emotive state (Borszcz, 1993, 1995). It has been suggested that it is the emotive state that becomes associated with the CS. Thus, during CS presentation at a later date, the emotive state is activated and this is measurable. The emotive state elicited by the CS produces behavioural and biological responses akin to a fear state. Thus it is plausible that the fear state produced by foot-shock is the same as that evoked by CS presentation.

In the following experiments the increase in acoustic startle reflex was used as an index of the central fear state. The main question asked was, is the central fear state elicited by footshock during the shock sensitization paradigm the same as that elicited by the CS after fear conditioning? To assess this, rats were classically conditioned and infused with a drug prior to CS testing. It was expected that drugs that suppressed immediate fear arousal produced by footshock would also prevent the expression of fear to CS presentation. This could be interpreted as a similarity between US and CS fear

expression, namely a central fear state. Conversely, drugs that fail to affect immediate fear arousal but do affect fear expression to CS may prevent expression through other mechanisms not related to the central fear state, i.e. mnemonic processes.

To investigate these similarities and differences, rats were infused with one of the following a $GABA_a$r agonist, a NMDAr antagonist, a non-NMDAr (AMPA) antagonist, a dopamine D1r agonist, a dopamine D2r agonist, or the protein synthesis inhibitor, anisomycin. For in-depth detail about the equipment, drugs and paradigm please consult the method section (see page 56), but a summary of the fear potentiated startle paradigm is presented here.

Rats were exposed to 60 noise trials on day 1, conditioned twice with 15 CS-US (light + footshock, 30 trials in total) at varied inter-trial intervals ranging between 60 and 180 seconds on day 2. The rats received a short test of 5 acoustic trials and 5 light plus acoustic trials to test for the effectiveness of fear conditioning on day 3. On day 4 the rats received a short 5 CS and US conditioning session in the morning to reduce potential extinction that the short L+N test the day prior could have elicited. The late afternoon session (the main test) comprised 20 acoustic startle stimuli, followed by drug infusion, followed by a further 20 acoustic startle stimuli to test for a drug effect on startle. This was directly followed by a fear test consisting of 10 acoustic startle stimuli and 10 CS + acoustic startle stimuli.

On the final day, day 5, rats were tested for fear with a 10 noise 10 light+ noise test. Acoustic startle data was collected for each group of rats during the four short manipulations; a 5 noise 5 CS+ noise to show that fear-conditioning was successful, a pre-drug/post-drug test to show the effect of the drug on acoustic startle, a drugged 10

noise, 10 CS + noise test to show the effect of the drug on fear-potentiated startle (FPS) and the final test 24 hours later to show that all the previous manipulations did not interfere with fear expression. The results of the four manipulations for each drug are presented in Figures 5.1-5.6.

Experiment 11: The role of GABA in fear expression during the fear-potentiated startle paradigm.

The previous section showed that immediate fear arousal by foot-shock could be blocked via enhancement of GABA inhibitory neurotransmission (van Nobelen & Kokkinidis, 2006). These results have been interpreted that GABA neurotransmission was necessary for the expression of the central fear state produced by the US (Blair, Sotres-Bayon, Moita, & LeDoux, 2005; van Nobelen & Kokkinidis, 2006). If this interpretation is correct, then GABA inhibition should also be involved in the expression of Pavlovian conditioned fear. Because it is postulated that the central fear state produced by a US is the same as the central fear state produced by a CS, it was expected that the infusion of a low dose of muscimol would inhibit the increase in startle amplitude associated with CS presentation. Functional inactivation of the amygdala, via high concentrations of muscimol, before a fear expression test has been shown to reduce freezing and place avoidance (Helmstetter & Bellgowan, 1994; Holahan & White, 2004; Muller, Corodimas, Fridel, & LeDoux, 1997; Wilensky, Schafe, & LeDoux, 2000), but it is unclear if a low concentration of muscimol prevents fear expression using the fear potentiated startle paradigm.

To examine this, 23 rats were surgically prepared of which 9 were rejected after histological verification. Two groups of rats were infused with either saline or 0.005μg

muscimol into the basolateral amygdala. The data of the experimental group are compared with the control group that received identical treatment but was infused with physiological saline. This control group is also compared to drugs used in experiments 12-16.

Figure 5.1 displays the mean startle amplitudes for the two drugs, saline (N=7) and 0.005µg muscimol (N=7) during the 4 manipulations. Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's brain atlas (1998).

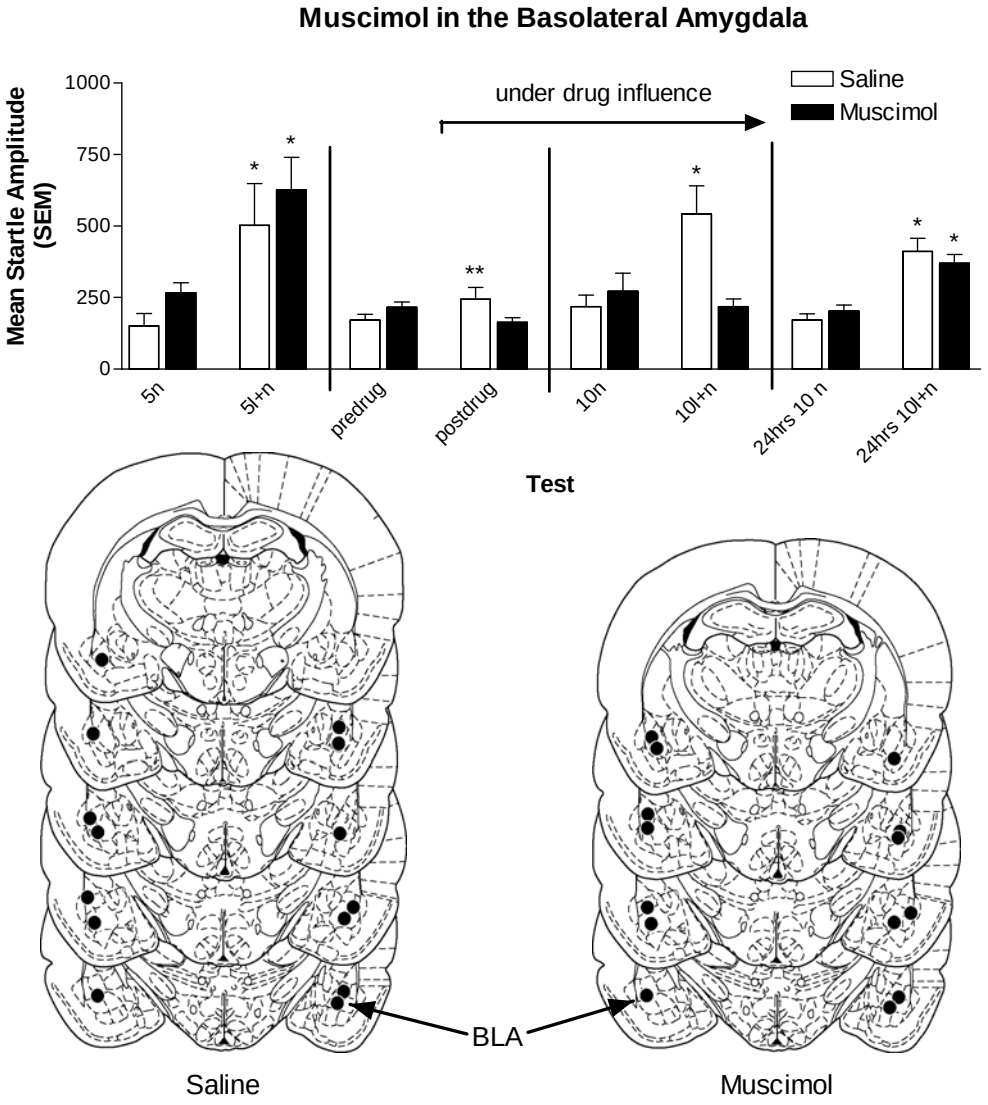

Figure 5.1: Effect of muscimol on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 0.005µg/µl muscimol (N=7) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with muscimol could not express fear during the main 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, $P<0.05$. ** denotes a significant increase in startle after saline infusion, $P<0.05$. Cannulae locations of the saline and muscimol groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson's (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.1 demonstrates that the infusion of a low dose of muscimol inhibited startle responses after CS presentation during the main fear test as compared to the saline group. Analysing the data collected during the main fear-potentiated startle test using a 2 x 2 repeated measures ANOVA revealed a significant drug x test interaction ($F_{1,12}$= 12.59, P< 0.004), and a significant main test effect ($F_{1,12}$ = 6.50, P<0.02). Simple effects analyses indicated that the saline group displayed increased startle amplitudes (F=18.60, P<0.001) while the muscimol group did not (F=0.49). The failure of startle augmentation seen in the muscimol group indicates that these rats were unable to demonstrate fear to CS presentation. This claim is supported by results with a muscimol dose 100 times higher (0.5μg) during which rats did not freeze to context or a tone CS (Maren & Holt, 2004; Muller, Corodimas, Fridel, & LeDoux, 1997). A high dose can have analgesic properties and cause functional inactivation, while a lower dose suggests that increasing the inhibitory function of the GABAnergic system is effective in the reduction of fear expression. This result was expected because the data in the previous section revealed that a low dose could block the central fear state during the shock sensitization (van Nobelen & Kokkinidis, 2006). This overlap suggests that the central fear state elicited by the US in the basolateral amygdala has a similar neural basis to the central fear state elicited during the CS presentation (Chi, 1965). However, it cannot be excluded that muscimol may interrupt mnemonic effects to CS presentation. For example, memory consolidation is spared after muscimol infusions once fear acquisition is completed (Wilensky, Schafe, & LeDoux, 1999, 2000). This indicated that muscimol does not affect neural plasticity in a similar manner to glutamate (Tizzano, Griffey, & Schoepp, 2002). And infusion prior to acquisition prevents fear expression at a later date (Helmstetter &

Bellgowan, 1994; Poremba & Gabriel, 1999; Wilensky, Schafe, & LeDoux, 2000). These results have been interpreted as a deficit in plasticity during memory formation, but not after, and thus argue against a mnemonic effect of GABA on fear expression. The failure of plasticity during acquisition is in line with results reported in this study, because, if GABA reduces the central fear state than plasticity cannot proceed, and hence no fear acquisition occurs. Moreover, if GABA reduces the central fear state then this may explain the reported deficit found here and elsewhere in fear expression to a CS (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997).

The drug effect reported here was not due to effects on the central amygdala, as Section 4 and 5 Experiments 19 and 25 showed that a small concentration of muscimol failed to suppress fear expression in both the shock sensitization and the fear-potentiated startle paradigms.

The startle deficit seen under drug influence was abolished twenty four hours later. As is illustrated in Figure 5.1, both groups showed significant increases in startle after CS presentation, and statistical analyses showed a main test effect ($F_{1,12}$=69.91, P< 0.000002). This manipulation suggests that the muscimol-induced fear deficit was of a temporary nature, and did not cause extinction. Additionally, the infusion procedure itself was not the reason for the deficit seen during the main fear test. This test also showed that fear was still present after the main fear test. Thus, it is unlikely that the rats failed to experience fear during the main test, but this was suppressed by the presence of muscimol.

The lack of fear expression under muscimol influence was not due to sensori-motor impairment. To determine if the drugs caused sensori-motor impairment a 2 (pre-drug-

post-drug) x 2 (drug) repeated measures ANOVA was performed and revealed a significant interaction ($F_{1,12}$=8.30, P<0.01). Simple effects analyses showed that the startle response in the saline group was marginally augmented (F=5.56, P< 0.03). Conversely, muscimol showed no effect (F=2.95, n.s.). The heightened reaction in the saline group suggests that the infusion procedure and/or the context of the chamber may have augmented the reactivity of the saline group. This effect was not seen in the muscimol group. More importantly, both groups did not show a significant decline in acoustic startle indicating that the drug infusion did not cause a sensori-motor impairment.

It could be argued that rats that failed to show fear during the main drugged fear test (i.e. the muscimol group) failed to have fear prior to testing. This is unlikely, because in the short fear test the rats showed a significant increase in startle magnitude after CS presentation. In fact, both groups showed significant fear in the short 5 noise, 5 light + noise test. A 2 (drug) x 2 (test) repeated measures ANOVA revealed a robust test effect ($F_{1,12}$=19,88, P<0.0007), indicating that both groups had significant fear prior to testing under the drug's influence.

Thus a low dose of muscimol not only attenuated fear arousal in the shock sensitization paradigm but also did so during the fear-potentiated startle paradigm. This similarity was predicted, as the immediate fear-arousal produced by unexpected foot-shock represents the emotional value of the US (van Nobelen & Kokkinidis, 2006). During fear-potentiated startle this same emotive value is recalled and is suggested to be the reason for the enhancement of startle expression (Borszcz, 1995; Borszcz & Leaton, 2003). The effectiveness of a low concentration of muscimol supports GABAergic tone

being part of the underlying mechanism involved in fear processing (Fanselow & Kim, 1992).

Experiment 12: The role of NMDA neurotransmission in fear expression during the fear-potentiated startle paradigm.

The next experiment investigated the role of the NMDAr antagonist AP-5 on fear expression. Glutamate neurotransmission is implicated in the formation of US/CS associations (Fanselow & Kim, 1994; Maren, Aharonov, Stote, & Fanselow, 1996; Maren & Fanselow, 1996; Walker, Paschall, & Davis, 2005), memory (LeDoux, 2000), and also in routine synaptic transmission (Faber, Delaney, & Sah, 2005; Farb & LeDoux, 1997; Li, Stutzmann, & LeDoux, 1996). Thus there are multiple functions for NMDA neurotransmission. Some research groups reported that AP-5 reduced fear expression (Fendt, 2001; Lindquist & Brown, 2004), while others did not (Campeau, Miserendino, & Davis, 1992; Miserendino, Sananes, Melia, & Davis, 1990). Various methods of fear testing were used, including freezing and startle behaviour.

AP-5 did not affect fear arousal caused by footshock during the shock sensitization paradigm and the results were interpreted as neither neural transmission involving NMDA nor long term potentiation being engaged. Therefore, it was likely that AP-5 would not affect fear expression and thus predicting an increase in startle magnitude after CS presentation.

To investigate this possibility, 12 rats were surgically prepared with bilateral cannulae aiming for the basolateral amygdala and trained and tested according to the FPS paradigm. After histological verification only 6 rats remained in the AP-5 group. This group was compared to the same saline group for which data was reported in the

muscimol experiment, Exp 11 (page 110). The drug dose for this paradigm and the shock sensitization paradigm was the same, namely 5µg/µg AP-5. The results depicted in Figure 5.2 shows the mean startle amplitudes of the AP-5 group (N=6) in comparison with the saline group (N=7). Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's (1998) brain atlas.

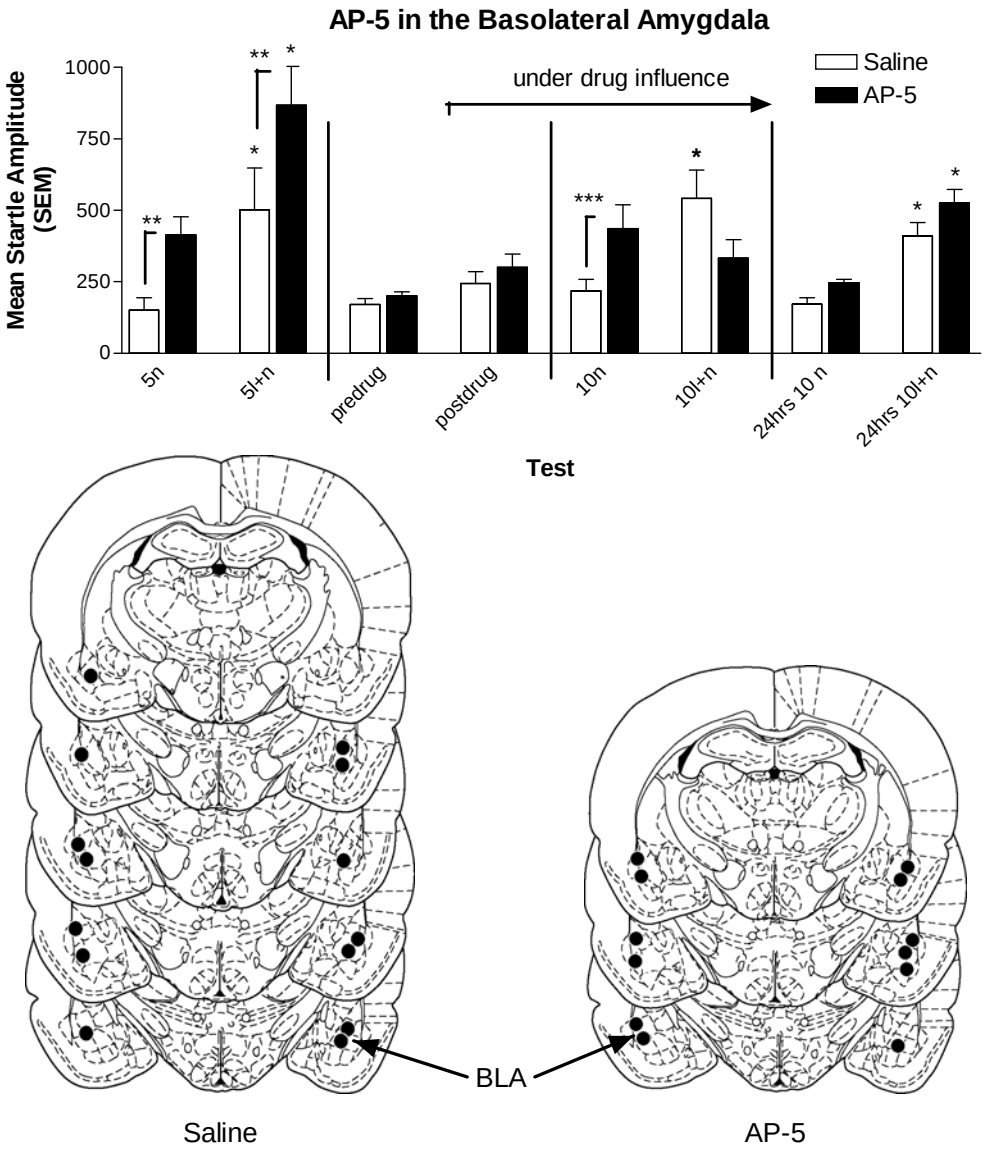

Figure 5.2: Effect of AP-5 on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 5µg/µl AP-5 (N=6) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with AP-5 could not express fear during the main 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, $P<0.05$. ** denotes a significant increase in startle between saline and the AP-5 groups during the 5n, 5l+n test, $P<0.05$. *** denotes a significant increase between saline and AP-5 during the 10n trials in the main fear test, $P<0.05$. Cannulae locations of the saline and AP-5 groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson's (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.2 illustrates that rats under influence of AP-5 were unable to exhibit an increase in startle responding after CS presentation, suggesting an inability to express fear. Despite the fact, that during the drugged 10 noise trials the AP-5 infused rats showed a significant elevation in response to noise as compared to saline ($F=6.02$, $P<0.03$), this difference was attenuated once the CS was presented. A 2 (test) x 2 (drug) repeated measures ANOVA yielded a significant interaction effect ($F_{1,11}=13,43$, $P<0.003$). Simple effects analyses showed that the saline group reacted with significant augmentation of startle to CS ($F=16.74$, $P<0.001$), the AP-5 group did not ($F=1.45$, n.s.).

These results are comparable to the failure in fear expression measured via freezing, fear potentiated startle, ultra sonic vocalization, eyeblink facilitation, defecation and analgesia that were blocked after the infusion of AP-5 prior to fear testing (Fendt, 2001; Lee & Kim, 1998; Lee, Choi, Brown, & Kim, 2001; Lindquist & Brown, 2004; Maren, Aharonov, Stote, & Fanselow, 1996). Furthermore, high (5.0µg) and low (0.625µg) concentrations of AP-5 significantly blocked the expression of conditioned defeat in hamsters (Jasnow, Cooper, & Huhman, 2004).

It could be argued that the rats in the AP-5 group were more reactive during the drugged 10 acoustic startle presentations and this may have masked the ability to respond to the CS presentation, i.e. a ceiling effect may have been reached which rendered rats physically unable to respond further. However, this was unlikely because rats did respond vigorously to the CS (as seen in the earlier 5 noise 5 CS test and in the later 24 hr test). Moreover, if AP-5 had no effect then the rats should have increased their responses to the CS presentation. In fact these were reduced.

This drug effect of inhibiting fear expression during CS presentation was abolished 24 h later- a 2 (test) x 2 (drug) repeated measures ANOVA yielded significant main drug and test effects ($F_{1,11}$=5.76, P<0.04 and $F_{1,11}$=66.32, P<0.000001, respectively). As illustrated in Figure 5.2, the drug effect found in the statistical analyses suggests that the AP-5 group was more reactive during both the 10 noise and 10 Light + noise tests, compared to saline. The test effect indicated that both groups responded more to the CS presentation as compared to the noise alone.

Furthermore, this test showed that AP-5 did not produce extinction (as tested 24 hours later) because the rats were capable of showing augmented acoustic startle after CS presentation. Finally, this test indicated that the infusion of AP-5 has a temporary effect on blocking fear expression, and that the previous manipulations of infusion and fear testing have not left detectable traces.

As NMDA neurotransmission is needed for excitatory synaptic transmission (Weisskopf & LeDoux, 1999) and the antagonist AP-5 could potentially have suppressed most neuronal activity and produced a form of ataxia. This ataxia may have been the reason for the failure of fear expression during the main fear test, but as illustrated during the pre-drug/post-drug test, this was not the case. AP-5 infusion had a small but insignificant effect on startle; this was measured by comparing the 20 noise pre-drug to the 20 noise post-drug. The startle amplitude was not reduced. Moreover, during the main fear test, these same rats showed an increase in startle amplitude, thus arguing against an ataxic effect.

As illustrated in Figure 5.2, there was a slight elevation in response to noise after drug infusion during the pre-drug/post-drug test. A 2 (test) x 2 (drug) repeated measures

ANOVA with the repetition on the test variable showed a main test effect ($F_{1,11}$=6.68, P<0.03). However, simple effects analyses revealed that the saline group was not affected by the saline infusion (F=2.60, n.s.), but the AP-5 group nearly reached significance (F=4.11, P=0.07). Thus, if AP-5 stimulates the rat during acoustic startle this was not augmented by CS presentation but instead diminished it thus lending support to the argument that AP-5 does indeed attenuate the ability to express fear to CS presentations.

A final argument could be that the rats did not experience fear prior to the infusion, but as can be seen in Figure 5.2 during the short fear test, both groups displayed significant fear. A 2 (test) x 2 (drug) repeated measures ANOVA ($F_{1,11}$=29.27, P< 0.0002), main test effect and a main drug effect ($F_{1,11}$=5.02, P<0.04) showed that overall the rats in the AP-5 group were more responsive during this test. Figure 5.2 depicts that, throughout the fear-potentiated startle paradigm the rats in the AP-5 group were more reactive overall then the saline group except when under drug influence in the main fear test during CS exposure.

An interesting finding is that while AP-5 did not influence fear expression during the shock sensitization paradigm, it did so during the Conditioned fear expression paradigm. This suggests that NMDAr are not involved in the physical or emotive properties of fear expression but in its cognitive elements. A similar conclusion was drawn by Lee, Choi, Brown and Kim (2001) who suggested that glutamate neurotransmission is involved in long-term potentiation which in turn is related to the formation and the recall of the US-CS association. Consistent with this is, that memory retrieval requires CREB phosphorylation and Fos expression which is akin to memory formation (Hall, Thomas, & Everitt, 2001). Memory formation requires NMDA

activation and can be prevented through the infusion of an NMDA antagonist such as AP-5 (Bauer, Schafe, & LeDoux, 2002). Thus, it is likely that memory retrieval is suppressed by AP-5 infusion. The NMDA receptor antagonist AP-5 prevents the receptor complex from functioning. The complex consists of more than one receptor subtype. Of these subtypes, the NR2B prevents fear acquisition (Rodrigues, Schafe, & LeDoux, 2001) and the NR2A attenuates the retrieval of auditory fear conditioning (Moriya et al., 2000) there supporting that AP-5' interference with fear expression as well as acquisition through a NMDA mechanism.

Experiment 13: The role of non-NMDA neurotransmission in fear expression during the fear potentiated startle paradigm.

Glutamate synaptic neurotransmission is partially mediated by AMPA receptors which are necessary for routine synaptic neurotransmission, formation and the expression of long term potentiation in the basolateral amygdala (Clugnet & LeDoux, 1990; Farb & LeDoux, 1997; Li, Stutzmann, & LeDoux, 1996). Section 2 showed that AMPA antagonism had no effect on immediate fear arousal to a US. The high dose-rate did not affect routine synaptic transmission or any form of fear acquisition/ expression.

Infusion of an AMPAr antagonist prior to fear acquisition blocked fear potentiated startle (Walker & Davis, 2002) and conditioned taste aversion (Yasoshima, Morimoto, & Yamamoto, 2000). This was interpreted as an acquisition effect. Additionally, the rate of fear acquisition could be enhanced via AMPA agonism (Rogan, Staubli, & LeDoux, 1997a), thus supporting the necessity of AMPA receptors for long-term potentiation (Izquierdo, 1994). Furthermore, AMPA neurotransmission is implicated in fear expression (Roesler et al., 1999) since both inhibitory avoidance and fear potentiated

startle are suppressed after AMPA receptor antagonism (Kim, Campeau, Falls, & Davis, 1993). Thus it was expected that CNQX would block fear expression during the fear-potentiated startle paradigm. To investigate this, 10 rats were surgically prepared and infused with a pharmacologically effective dose of CNQX. The dose rate of 5 μg/μl was used, which is identical to the other non-NMDA experiments in this thesis. This dose was based on findings that it effectively blocked the acquisition and expression of fear-potentiated startle (Kim, Campeau, Falls, & Davis, 1993; Walker & Davis, 2002). After histological verification 6 rats were eliminated and excluded from the analyses. The data was compared to the saline group for which the data was reported in Experiment 11 (muscimol).

Figure 5.3 shows the comparison between the saline (N=7) control group and the CNQX (N=7) group during the 4 manipulations. Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's (1998) brain atlas.

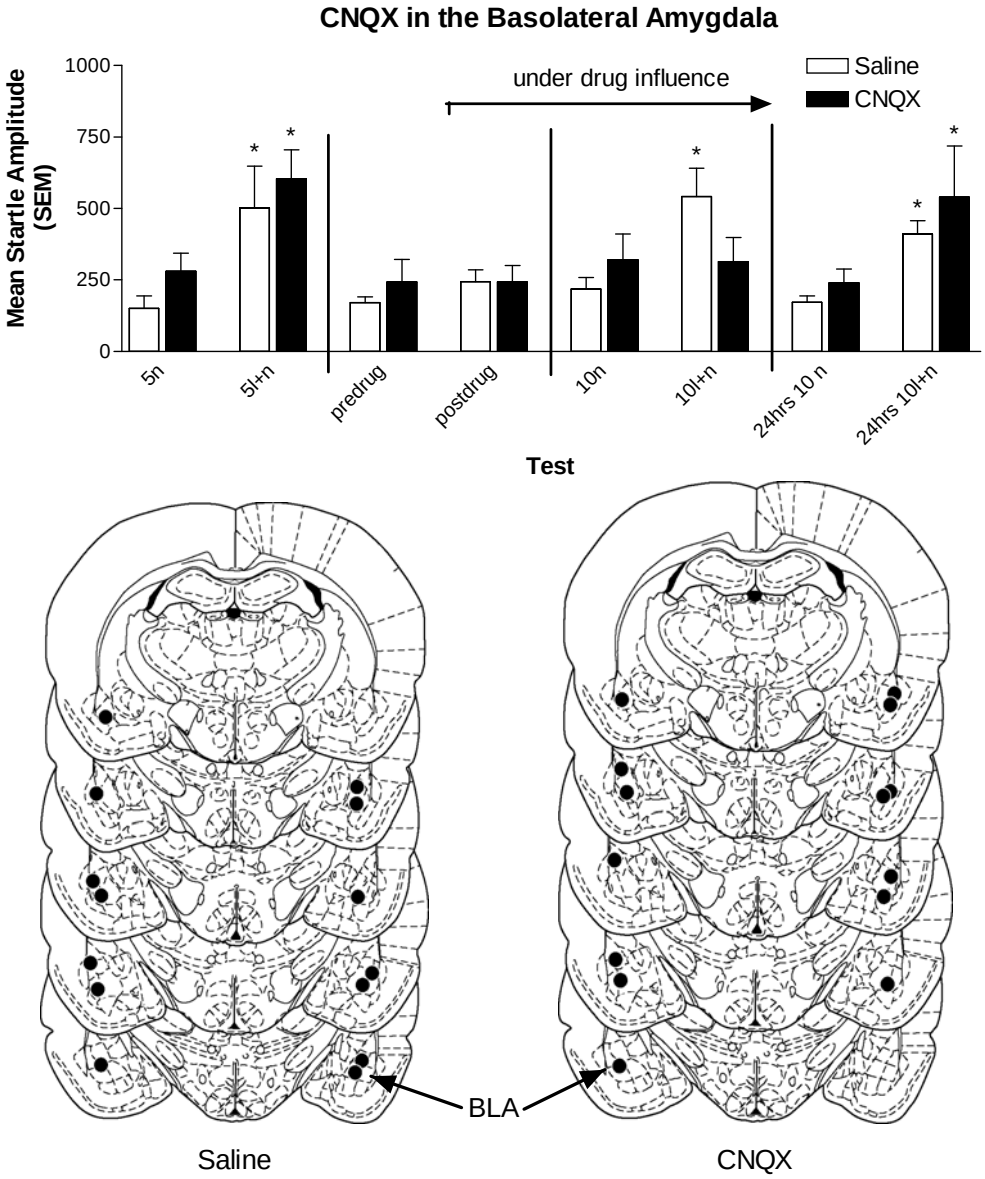

Figure 5.3: Effect of CNQX on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 5µg/µl CNQX (N=7) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with CNQX could not express fear during the 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, $P<0.05$. Cannulae locations of the saline and CNQX groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson's (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.3 illustrates that, during the main fear test the CNQX group failed to show augmented acoustic startle after the CS presentation. A 2 (test) x 2 (drug) repeated measures ANOVA, yielded an interaction effect ($F_{1,12}$=8.33, P<0.01) and a main test effect ($F_{1,12}$=7.63, P<0.01). Simple effects analyses showed that the saline group could exhibit augmented acoustic startle, F=15.69, P<0.001 while the CNQX group could not (F=0.007, n.s.) thereby confirming findings that AMPAr antagonism clearly attenuates visual, auditory, olfactory, and gustatory CS fear expression (Kim, Campeau, Falls, & Davis, 1993; Walker, Paschall, & Davis, 2005; Yasoshima, Morimoto, & Yamamoto, 2000). These authors concluded this was not due to suppression of responses during testing but to inhibition of the AMPA receptors necessary for fear expression.

The effect of CNQX on fear expression was absent 24 hours later, when both groups showed significant CS-induced enhancement of acoustic startle, [(2 (test) x 2 (drug) repeated measures ANOVA, main test effect ($F_{1,12}$=12.17, P<0.004)], thereby showing that fear suppression seen during the fear expression test was of temporary nature. This manipulation also provided evidence that the infusion procedure and CNQX had no lasting effects.

It can also be shown that the attenuated fear responses under the influence of CNQX were not caused by ataxia in the rats. By comparing the pre-drug/post-drug data it is clear that infusion of CNQX had no influence on the acoustic startle response itself. Both the saline and the CNQX groups were unaffected by the infusion of vehicle or vehicle and drug. This was analysed with the 2 (test) x 2 (drug) repeated measures ANOVA, which showed no significant results (P=0.17).

The inhibition of fear-potentiated startle after the infusion of CNQX was not due to a failure of fear acquisition prior to testing. The short fear test distinctly showed that both groups displayed significant fear during this test, [(2(test) x 2(drug) repeated measures ANOVA, repetition on the test variable, main test effect ($F_{1,12}$=27.09, P<0.0002)].

AMPA receptors are involved in 'fast' synaptic transmission; they function via depolarization of the postsynaptic terminal (McKernan & Shinnick-Gallagher, 1997). One of their main roles is in memory formation and recall (Izquierdo, 1994). Results from Section 2, Experiment 7 (page 86), showed that the AMPA receptors were not involved in foot-shock-enhanced acoustic startle, and thus not in a central fear state produced by footshock. But, they are involved in Conditioned fear potentiated startle, and may act via a mnemonic route, including attention and recall of CS (Izquierdo et al., 1993).

Experiment 14: The role of protein synthesis inhibition in fear expression during the fear-potentiated startle paradigm.

The next experiment in this section investigated the role of anisomycin on fear expression during CS presentation. During a CS/US pairing long-term potentiation occurs in which NMDA receptors and consequently protein syntheses are involved (Miserendino, Sananes, Melia, & Davis, 1990; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999). De novo protein synthesis is necessary for memory formation/ consolidation (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Schafe & LeDoux, 2000) and has been implicated in 'memory reconsolidation' (Duvarci & Nader, 2004; Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006).

The novo protein synthesis inhibitor anisomycin had no effect during the shock-augmented startle as shown in Section 2, Experiment 8 (page 92). This indicated that immediate fear arousal produced by footshock was not mediated by protein synthesis. Since protein synthesis has a role in fear learning, expression and consolidation (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Schafe & LeDoux, 2000; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999), it was necessary to investigate the role of anisomycin during the fear-potentiated startle paradigm. Because exposure to the CS during the main fear test would need retrieval of the central fear state and conceivably 'reconsolidation' of the CS and fear arousal, the potential for a fear expression deficit was likely. To investigate this, 12 rats were surgically prepared and infused with 80 µg anisomycin per side during the drugged section of the FPS paradigm. Figure 5.4 illustrates the comparison between rats in the saline (N=7) control group (from Experiment 11) and rats in the anisomycin (N=7) group during the 4 manipulations. After histological verification 5 rats were eliminated and excluded from the analyses. Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's (1998) brain atlas.

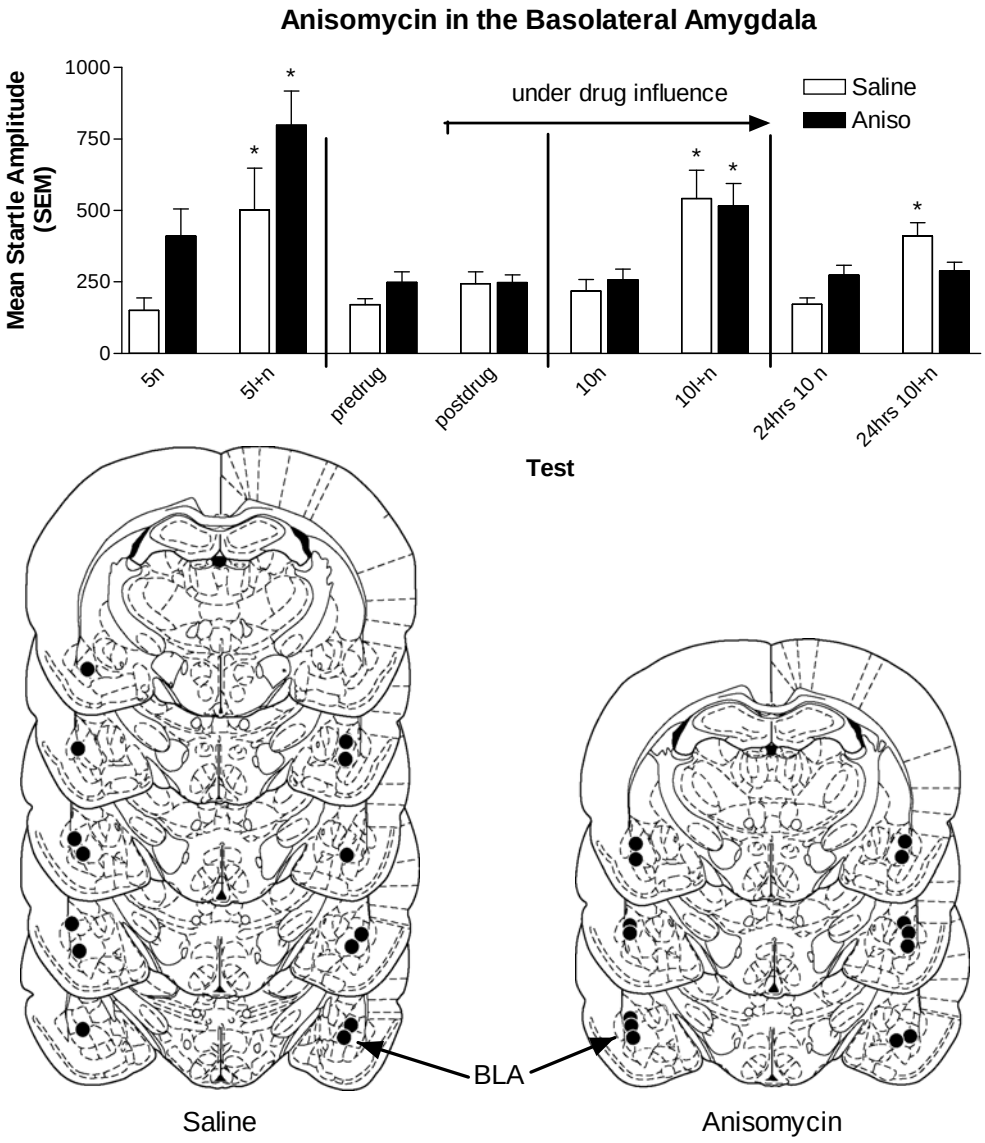

Figure 5.4: Effect of anisomycin on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 80µg/µl anisomycin (N=7) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with anisomycin could not express fear during the 24 h10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, $P<0.05$. Cannulae locations of the saline and anisomycin groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson's (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.4 illustrates that both the saline and the anisomycin group displayed significantly augmented acoustic startle to CS presentation during the main fear test [(2(test) x 2(drug) repeated measures ANOVA, main test effect, $F_{1,12}$=20.64, P<0.0006)]. These results can be interpreted as both groups showing significant fear to the CS. The infusion of anisomycin into the basolateral amygdala failed to suppress fear expression.

The most interesting finding illustrated in Figure 5.4 is that the rats in the anisomycin group, could not express fear during the fear test 24 hours later. This was confirmed with statistical analyses that showed rats treated with anisomycin during the main fear test could not show augmented startle responses to a CS in the '24 hour later' test. A 2(test) x 2(drug) repeated measures ANOVA revealed a significant interaction ($F_{1,12}$=23.84, P<0.0003) and a main test effect ($F_{1,12}$=30.69, P<0.0001). Simple effects analyses showed that the saline group still showed augmented startle (F=54.31, P<0.000001) while the anisomycin group did not (F=0.21, n.s.).

These results are in agreement with work published by Nader, Schafe and Le Doux (2000a) who studied freezing during a reconsolidation test. Rats were tested with CS exposure and directly afterwards infused with anisomycin. Twenty four hours later the same rats were presented with the CS and they failed to display freezing behaviour thus indicating a lack of fear. Similar to the results reported here, the infusion of anisomycin had no visible behavioural effects on immediate recall but affected expression 24 h later (Debiec, Doyere, Nader, & LeDoux, 2006). However, there were two notable differences between the works of Nader et. al. (2000a) and this fear-potentiated startle paradigm. In Experiment 14 anisomycin was infused before recall, while Nader et. al. (2000) infused the drug directly after recall. More importantly were the differences in strength of

conditioning and the measure of fear expression. In Experiment 14, rats were exposed to a total of 35 CS/US conditioning trials, and were tested for fear with acoustic startle, while Nader et. al. (2000) used a single CS/US trial as a conditioning paradigm and used freezing behaviour as the measure of fear. Longer conditioning leads to a stronger association and potentially to more difficulty in disrupting connections. It has been shown that stronger training protocols affect anisomycin sensitivity differently from weaker protocols (Bourtchouladze et al., 1998). In addition, fear potentiated startle uses a slightly different neural circuit than freezing (Borelli, Gargaro, dos Santos, & Brandao, 2005; McNisch, Gerwirtz, & Davis, 1997). Thus both freezing and fear potentiated startle circuits were affected by anisomycin.

Experiment 14 showed that the infusion of anisomycin before fear expression affected later fear potentiated startle. A possible explanation is that memories directly related to the CS become labile and these are sensitive to protein synthesis (Rodriguez-Ortiz, De la Cruz, Gutierrez, & Bermudez-Rattoni, 2005). However, if the memory is not reactivated under the influence of anisomycin, it is not affected 24 h later (Debiec, Doyere, Nader, & LeDoux, 2006; Milekic & Alberini, 2002; Nader, Schafe, & LeDoux, 2000b). Additionally, the older the memory the less likely it was to be affected by anisomycin during reconsolidation (Milekic and Alberini, 2002). Thus, more then 14 days after training, memory becomes less easily affected by anisomycin. Since all rats were tested within 3 days of training they were more likely to be affected by the infusion of anisomycin.

An alternative explanation is that anisomycin had caused extinction. Extinction in this case is the acquisition of new associations between the CS and startle, whereby the

CS now predicts a safe situation and thus a reduction in fear expression during later tests. But since anisomycin can disrupt learning of new associations it is unlikely that new associations were formed between the CS and the new non-reinforced trials (Duvarci & Nader, 2004). This was not tested by further fear testing, but it has been shown that the fear reduction noticed after anisomycin infusion is temporary depending on the tests used (Duvarci & Nader, 2004; Lattel & Abel, 2004). If the deficit shown was extinction then reinstatement trials should reinstate the fear. Reinstatement is the presentation of some un-cued footshocks that reinstate measurable fear arousal to a previously extinguished CS/US association. However, reinstatement does not appear to recover the amnesia induced by anisomycin (Duvarci & Nader, 2004).

A final explanation could be that anisomycin had no effect at all and that the rats underwent extinction during the main test. This was unlikely because during all 6 experimental groups, including the saline control group, the rats showed few signs of extinction, as measured by the last significant fear test. The question remains: what has happened to the central fear state?

Figure 5.4 illustrates that the infusion of anisomycin into the basolateral amygdala had no effect on startle expression. In fact the startle amplitudes were very similar across the pre-drug/post-drug test. Statistical analyses showed a non-significant result, [2 (test) x 2 (drug) repeated measures ANOVA, n.s.]. This was interpreted as anisomycin not affecting acoustic startle.

Finally, both groups of rats in the saline and the anisomycin group showed significant fear potentiated startle during the short test, [2(test) x 2(drug) repeated measures ANOVA, main test effect ($F_{1,12}$=28.12, P<0.0001)]. The anisomycin group did

show elevated reactivity to the acoustic startle during both noise and CS + noise presentations but this did not reach significance ($F_{1,12}$=4.09, P=0.07).

In summary, although the infusion of anisomycin prior to fear recall did not affect fear expression immediately, it did affect fear expression 24 hours post-infusion. These findings add to existing knowledge that anisomycin infused prior to recall, as opposed to after recall (Nader, Schafe, & LeDoux, 2000b), can attenuate fear expression to a CS presented 24 hours later. This effect can be found using fear-potentiated startle and also freezing behaviour as a measure of fear (Nader, Schafe, & LeDoux, 2000b).

Experiment 15: The role of a dopamine D1 agonist, SKF 38393, in fear expression during the fear-potentiated startle paradigm.

Dopamine can act as a neuro-modulator. This is partially dependent upon the pre and postsynaptic characteristics of the neurons. Furthermore, it has been reported that dopamine has a predominantly inhibitory function (Ben-Ari & Kelly, 1976) which can be activated via direct inhibition and via interneurons located in the basolateral amygdala (Kroner, Rosenkranz, Grace, & Barrionuevo, 2005; Rosenkranz & Grace, 1999). The dopamine system in the basolateral amygdala is part of the mesoamygdaloid system that originates in the ventral tegmental area and is involved in fear responding (Gelowitz & Kokkinidis, 1999; Waddington Lamont & Kokkinidis, 1998; Young & Rees, 1998). Long-term potentiation can be disrupted by changes in dopamine D1 levels (Kerr & Wickens, 2001). Intraperitoneal and basolateral injections of the D1 antagonist, SCH 23390, blocked the acquisition of a CS/US association, but this was not due to decreased shock reactivity or attenuation of the immediate fear arousal produced by footshock (de Oliveira, Reimer, & Brandao, 2006; Greba & Kokkinidis, 2000). The D1 antagonist

attenuated the acquisition of second order conditioning. Conversely the D1 agonist had no effect (Nader & LeDoux, 1999a, 1999b). Furthermore, the D1 antagonist SCH 23390 could also block fear expression during a fear potentiated startle paradigm (Waddington Lamont & Kokkinidis, 1998). Together a role for dopamine D1r antagonists in fear processing exists, but the role of the D1 agonist in the basolateral amygdala has received little attention.

In Section 2, Experiment 9 rats showed that the D1 agonist had no effect on immediate fear arousal caused by US presentation. This failure does not automatically indicate that D1 receptor agonism may not influence fear expression to explicit cues, because fear expression to an explicit cue involves a cognitive component. It has been reported that dopamine can inhibit neuronal firing patterns (Loretan, Bissiere, & Luthi, 2004) and these may be required for fear expression to explicit cues. To investigate the role of the D1 agonist, 11 rats were surgically prepared and infused with 4 µg/µl SKF 38393. After histological verification 4 rats were excluded, mainly for misplaced cannulae, and were not used for statistical analyses. Figure 5.5 shows the mean startle amplitudes during the 4 manipulations. Rats in the SKF 38393 (N=7) group were compared to the saline group (N=7) from Experiment 11. Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's (1998) brain atlas.

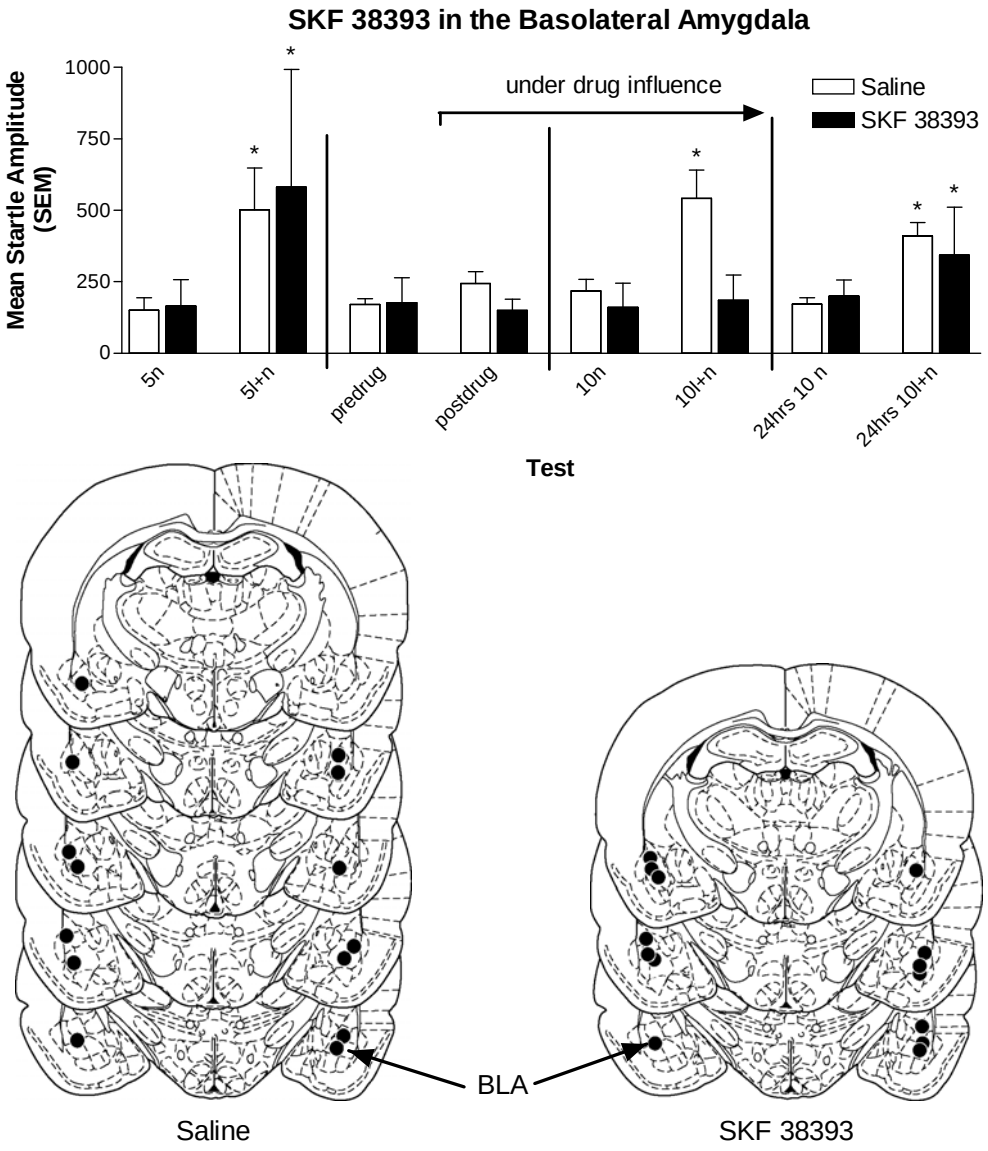

Figure 5.5: Effect of SKF 38393 on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 4µg/µl SKF 38393 (N=7) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with SKF 38393 could not express fear during the 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, P<0.05. Cannulae locations of the saline and SKF 38393 groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.5 shows that rats under the influence of SKF 38393 did not express fear during the main fear potentiated startle test. No significant differences were found between acoustic startle responses to the 10 noise and 10 CS plus noise trails. A 2(test) x 2 (drug) repeated measures ANOVA revealed a significant interaction ($F_{1,12}$=9,61, P<0.009). Simple effects analyses showed that the saline group had significant fear-augmented startle amplitudes while the SKF 38393 group did not (F=22.5, P<0.0004, F=0.13, n.s. respectively). These results were interesting as the D1 antagonist SCH 23390 has been shown to also block fear potentiated startle (Waddington Lamont & Kokkinidis, 1998). However, a similar contradictory result was reported by Zarrindast Rezayof, Sahaei, Haeri-Rohani and Rassouli (2003); they noticed that the infusion of either SKF 38393 or SCH 23390 into the central amygdala could block the expression of morphine-induced place preference. This suggests that either an excess or a deficiency of dopamine may interfere with fear expression. This was supported by in vitro analyses of lateral amygdala slices; here the addition of dopamine increased inhibitory postsynaptic currents (Loretan, Bissiere, & Luthi, 2004) indicating that dopamine is capable of inhibiting neuronal firing and thus may contribute to the inhibition of fear expression seen in the fear potentiated startle test.

The inhibitory effect of SKF 38393 was absent twenty four hours later. Both the control and the SKF 38393 groups displayed significant increases in startle amplitude during acoustic startle after CS presentation. Statistical analyses (2(test) x 2 (drug) repeated measures ANOVA) showed a main test effect ($F_{1,12}$=25.51, P< 0.0002), and the simple effects demonstrated that both groups experienced significant fear during the un-drugged state; saline (F=20.02, P<0.0007) and SKF 38393 (F=7.11, P<0.02). The return

of fear expression after SKF 38393 infusion indicates that the effect was of a temporary nature and that the infusion procedure did not permanently damage the functionality of the basolateral amygdala.

Arguably, SKF38393 may affect the sensori-motor response to noise thus affecting the ability express fear during the fear-potentiated startle test. A 2 (test) x 2 (drug) repeated measures ANOVA revealed a nearly significant interaction ($F_{1,12}$=4.36, P=0.058). As can be seen in Figure 5.5, with the pre-drug /post-drug test, the saline group showed a slight elevation in reactivity after infusion whereas conversely, the SKF38393 group did not. This indicates that SKF 38393 did not affect startle responses in a positive or negative manner.

In addition, the lack of fear expression during the main fear test could not be attributed to rats not experiencing fear, as both groups showed significant fear during the short 5 noise 5 light+ noise tests. A 2 x 2 repeated measures ANOVA showed a main test effect ($F_{1,12}$=18,50, P<0.001).

The suppression of fear is unlikely to be caused by the infusion of SKF 38393 affecting other amygdaloid areas, particularly the central amygdala; see Section 5 Experiment 26, in that experiment SKF 38393 significantly increased fear expression as compared to saline. Even though the concentration of SKF 38393 was relatively high (4 µg/µl) the rats only received 2µg/ 0.5µl per side, and as shown earlier, this concentration did not affect immediate fear arousal produced by footshock (see Exp 9).

The results of the four manipulations showed that both groups of rats experienced fear prior to the main drugged fear-potentiated startle test and also afterwards, but the SKF 38393 group was unable to express fear during the main fear test while under

influence of SKF 38393 (unlike the saline group). This effect was not due to suppression of the acoustic startle response by SKF38393.

Intraperitoneal injections of the D1 agonist, SKF 38393, or the D1 antagonist, SCH 23390, prior to fear testing had no effect on freezing behaviour (de Oliveira, Reimer, & Brandao, 2006; Inoei, Izumi, Maki, Muraki, & Koyama, 2000). Conversely, Greba and Kokkinidis 2000 observed reduced fear potentiated startle after intraperitoneal injections and intra-amygdalar infusion of SCH 23390 (Waddington Lamont & Kokkinidis, 1998). Nader and Ledoux (1999) noticed that infusion of SKF 38393 into the basolateral amygdala did not affect second-order conditioning, as measured by freezing responses. This creates problems if a D1 agonist can suppress fear expression during recall, as shown above; then how do the rats learn in the second-order paradigm during which the CS2 predicts presentation of CS1? Because, if the rats are unable to express fear during CS1 presentation then the CS1 followed by CS2 will not form an association that at a later date will elicit freezing to CS2 presentation. A possible answer is that there is a marked difference between freezing and startle behaviour. Both behaviours measure fear but via different anatomical pathways (Borelli, Gargaro, dos Santos, & Brandao, 2005; McNisch, Gerwirtz, & Davis, 1997). The results also indicate that the drug effect was not due to the suppression of fear arousal (because immediate fear arousal by footshock was not affected), but may have been due to more inhibition of the neural firing responsible for a mnemonic effect (Greba & Kokkinidis, 2000).

Experiment 16: The role of a dopamine D2 agonist, quinpirole, in fear expression during the fear potentiated startle paradigm.

D2 dopamine receptor actvity in the basolateral amygdala has received a lot of attention. It has been reported that D2 receptors are mainly involved in fear expression. (de Oliveira, Reimer, & Brandao, 2006). However, there is also evidence that intraperitoneal injections (i.p.) and basolateral infusion of quinpirole decreases the acquisition of first and second-order conditioning (Greba, Gifkins, & Kokkinidis, 2001; Nader & LeDoux, 1999a). Furthermore, i.p. injections could suppress fear expression as measured by freezing and also fear-potentiated startle (de Oliveira, Reimer, & Brandao, 2006). As evident in Section 2 Experiment 9, the results of quinpirole infused into the basolateral amygdala during the shock sensitization paradigm were ambiguous. That is, the rats were very responsive to noise after infusion but this was not significantly augmented by foot-shock. Since the post shock response was similar to saline it was concluded that a ceiling effect had operated, and that quinpirole did not affect immediate fear arousal produced by footshock. Together, a case for D2 dopamine in fear expression exists but this has not yet been explored via infusion of quinpirole into the basolateral amygdala. To investigate this, 10 rats were surgically prepared and infused with 3.0 µg/µl quinpirole into the basolateral amygdala. After histological verification 4 rats were excluded and not used in statistical analyses. The data were compared to the results of the saline group from Experiment 11.

Figure 5.6 shows the mean startle response of rats infused with either saline (N=7) or quinpirole (N=6) into the basolateral amygdala during the drugged and un-drugged

manipulations. Cannulae locations in the basolateral amygdala of the drug groups are depicted on representative sections taken from Paxinos and Watson's (1998) brain atlas.

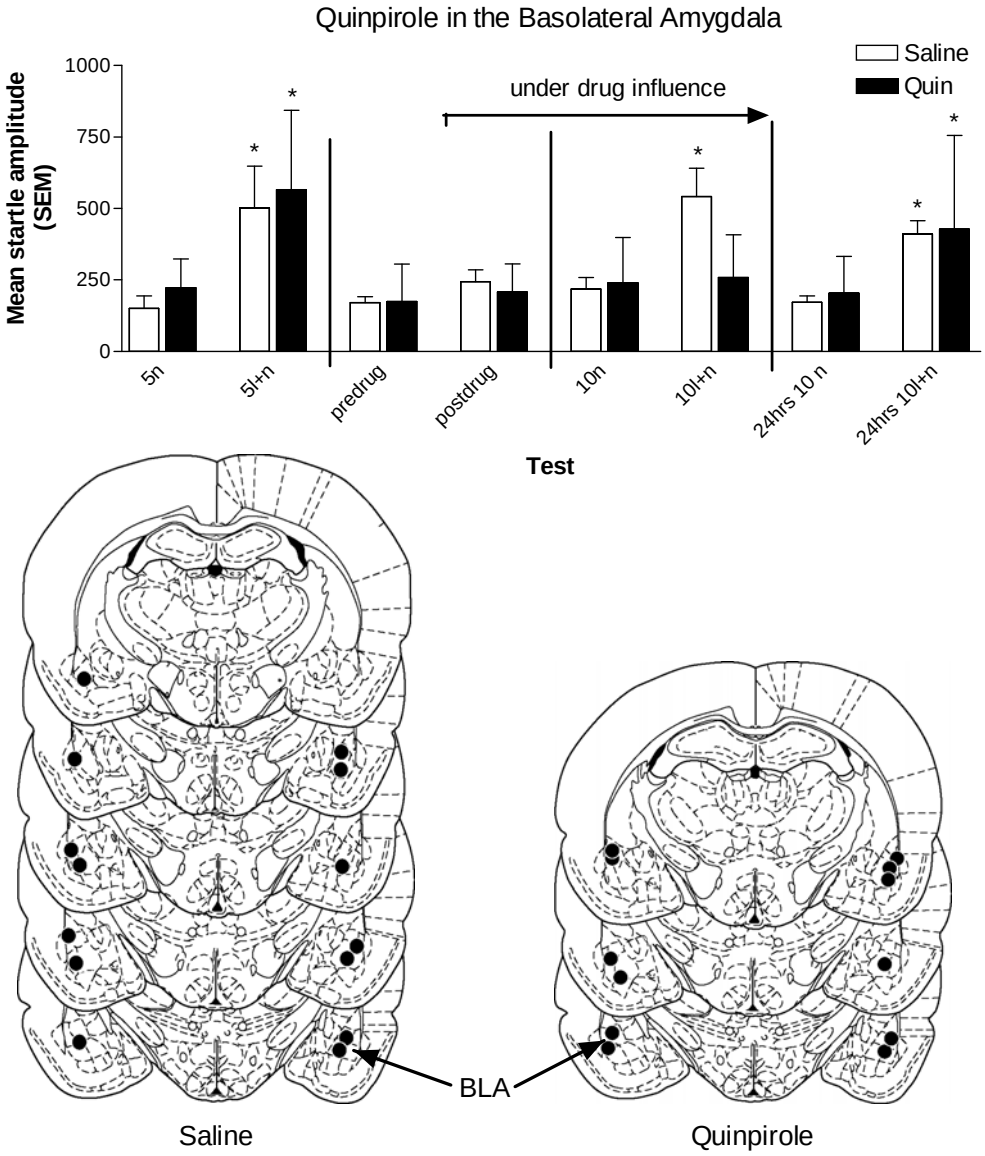

Figure 5.6: Effect of quinpirole on fear potentiated startle expression in the basolateral amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 3µg/µl quinpirole (N=6) after the pre-rug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with quinpirole could not express fear during the 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses after CS presentation, within each drug group, $P<0.05$. Cannulae locations of the saline and SKF 38393 groups depicted on schematics (Figs. 28-32) adapted from the Paxinos and Watson (1998) rat brain atlas. BLA, basolateral amygdala.

Figure 5.6 illustrates that during the drugged fear test rats under influence of quinpirole failed to show a fear-potentiated startle response. A 2 (test) x 2 (drug) repeated measures ANOVA, revealed a significant interaction ($F_{1,11}$=7.96, P<0.01). Simple effects analyses showed that contrary to the quinpirole group, the saline group showed significant acoustic startle responses to CS presentation (F=19.37, P<0.001; F=0.05, n.s. respectively). These observations increase knowledge about effects of systemic quinpirole application on freezing and fear-potentiated startle (de Oliveira, Reimer, & Brandao, 2006). The basolateral amygdala seems to be a locus for D2 activation during fear expression to a CS. As results from section 2 Experiment 9 indicated that quinpirole did not affect immediate fear arousal, it was more likely to have been a failure of the retrieval mechanism that prevented fear expression. These findings are consistent with results reported by Nader and LeDoux (1999) who concluded that systemic injections of quinpirole blocked the retrieval of the CS-US association (de Oliveira, Reimer, & Brandao, 2006).

This fear-blocking effect was not long-term because, 24 h later, the quinpirole group showed similar startle amplitudes to the CS as the control group in that both groups showed significant fear. A 2 (test) x 2 (drug) repeated measures ANOVA revealed a significant main test effect ($F_{1,11}$ =25.32, P<0.0003). These results also indicate that the infusion procedure did not disrupt the ability to express fear to a CS presentation.

It could be argued that quinpirole may have produced sensori-motor impairments. However, Figure 5.6 illustrates that quinpirole did not produce ataxia during the drugged state. Comparing the pre-drug to the post-drug startle data with a 2 (test) x 2 (drug) repeated measures ANOVA yielded no significant results, thus showing that the

suppression of fear during the FPS test was not due to the inability to respond to acoustic startle. Moreover, the rats certainly experienced fear prior to drug infusion because significant fear arousal was evident during the short un-drugged fear test. According to a 2 (test) x 2 (drug) repeated measures ANOVA, there was a significant main test effect ($F_{1,11}$=25.37, P<0.0003). Both the saline and the quinpirole group showed significant increases between acoustic startle before and after CS presentation.

The results showed that quinpirole blocks fear-potentiated startle to a CS and that this was not due to the inability to respond to noise or that the rats did not experience fear prior testing. The rats were also able to demonstrate significant fear during the final test indicating that the previous manipulations did not permanently affect the fear response.

The combined results of the shock sensitization and fear-potentiated startle paradigm suggest that D2 receptors are involved in fear expression but are more likely to be a mnemonic rather than an affective outcome. Similar conclusions have been drawn by Greba, Gifkins and Kokkinidis 2001, who suggested that dopamine is involved in processes related to retrieval of emotional memories. Furthermore, dopamine increases interneuron firing rate but does not affect GABAergic tone (Kroner, Rosenkranz, Grace, & Barrionuevo, 2005). Thus, even though both GABA and dopamine prevent fear expression this is due to different mechanisms.

Summary of results

In this section the main findings were 1) Experiment 11 showed the inhibitory effect of a low concentration of muscimol on fear expression. This was most likely due to inhibition of the central fear state. 2) Experiments 12 and 13 showed that both the glutamate antagonists prevented the expression of fear via an inhibition of excitatory

neural transmission. 3) Experiment 14 showed that the infusion of a protein synthesis inhibitor could prevent fear expression after 24 hours, but not directly after infusion. This was attributed to a reconsolidation effect. 4) Experiments 15 and 16 showed that both the D1 and D2 agonists could prevent fear expression to an explicit CS. This was ascribed to a failure in the mnemonic mechanism.

RESULTS SECTION 4: Experiments 17-21.

Analyses of footshock-elicited fear behaviour in the Central amygdala

The central amygdala receives most of its US and CS information from the basolateral amygdala (LeDoux, Iwata, Cicchetti, & Reis, 1988; Sah, Faber, Lopez de Armentia, & Power, 2003) and in turn projects this information to various areas responsible for fear expression. Regions such as the nucleus reticularis pontis caudalis, are responsible for startle, (Koch & Schnitzler, 1997; Rosen, Hitchcock, Sananes, Miserendino, & Davis, 1991), and the central gray area is responsible for freezing behaviour (Fanselow 1991). The older theory, that the central amygdala is a step along the fear expression pathway has been challenged by Paré, Quirk and LeDoux 2004. They suggested that the central amygdala directly receives US and CS information, and is thus potentially a site for plasticity. If information about the US arrives at the central amygdala it would be valuable to know what type of information this is. Borszcz (1993, 1995) suggested that the sensory characteristics of foot-shock include a stimulus-response relationship such as pain and spinal reflexes, and an affective-motivational response which may be involved in the stimulus-fear association. Results from Sections 2 and 3 showed that GABA neurotransmission in the basolateral amygdala was involved in the affective-motivational response (van Nobelen and Kokkinidis 2006; Blair, Sotres-Bayon, Moita and LeDoux 2005). Could this mean that the central amygdala is perhaps involved in stimulus-response elements, such as pain and the spinal reflex?

The central amygdala plays an important role in pain perception. For example, lesioning the central amygdala blocked enhanced vocalization, a measure of pain perception (Borszcz & Leaton, 2003; Crown, King, Meagher, & Grau, 2000), shock sensitization of startle and shock reactivity during footshock (Hitchcock, Sananes, & Davis, 1989). Additionally, intraperitoneal injections of morphine or buspirone reduced the shock sensitization effect but not baseline startle; the authors suggested a nociceptive effect, (Chen, Ho, & Liang, 2000). Furthermore, direct nociceptive tracts from the spinal cord arrive at the central amygdala and are part of the spinoparabrachial path (Almeida, Roizenblatt, & Tufik, 2004). In effect this means that the central amygdala has the potential to enable perception of pain caused by footshock.

Thus it is important to analyse not only the drug effect on post-shock acoustic startle responses but also the reactivity to footshock itself. This is, because a reduction in shock-sensitized startle responses could suggest a potential inability to perceive pain. Conversely, a reduction in foot-shock reactivity itself may represent a spinal-reflex component or a combination of reflex and pain perception. To evaluate this, rats were cannualized aiming for the central amygdala. They received a drug infusion after the first 20 acoustic startle responses on day 2 to compare the drug effect on startle reflex and startle responses after foot-shock application. The paradigm, drugs and procedures were identical to those used when investigating the shock sensitization effect in the basolateral amygdala (Results Section 2).

A summary of the shock sensitization paradigm is as follows:

On day 1 rats were base-lined with 3 X 20 white noise bursts at 91, 95, 99 dB, at 20 seconds inter stimulus intervals (ISI). On day 2, rats received 20 white noise bursts (30 s

ISI), infused with drug, exposed to 20 white noise bursts (30 s isi), 10 foot-shock (10 s isi), followed by 20 white noise bursts (30 s isi). Further detail can be found in the method section on page 53.

Experiment 17: The role of glutamate antagonists in fear arousal produced by footshock in the Central amygdala.

The latest reports suggest that long-term potentiation occurs in the central amygdala and that glutamate neurotransmission (Goosens & Maren, 2003) and de novo protein synthesis (Bahar, Samuel, Hazvi, & Dudai, 2003) are required for this. Furthermore, the central amygdala plays an important role in pain perception (Manning, Martin, & Meng, 2003), which is modulated by various types of glutamate neurotransmission (Ikeda, Takahashi, Inoue, & Kato, 2007; Neugebauer & Li, 2003; Neugebauer, Li, Bird, Bhave, & Gereau, 2003). Both auditory and contextual conditioning can be prevented via inhibiting glutamatergic neurotransmission (Goosens & Maren, 2003). It was not clear if this was due to rats not perceiving footshock or due to a failure of long term potentiation induction. If glutamatergic inhibition prevents long term potentiation then the infusion of AP-5 or CNQX should not affect the shock sensitzation paradigm in a similar fashion to results reported in Section 2 whereby glutamate inhibition in the basolateral amygdaloid failed to affect shock sensitization. Alternatively, if glutamate neurotransmission is involved in pain perception, the infusion of glutamatergic antagonists may prevent the shock sensitization effect.

To examine the effects of glutamate antagonists on shock sensitization, 30 rats were surgically prepared and infused with AP-5 or CNQX and compared to the saline group. Histological verification eliminated 3 rats from the CNQX group and 2 rats each from the

AP-5 and control group. Figure 6.1 shows the mean startle amplitudes before and after drug infusion and after footshock. Cannulae placements in the central amygdala of all groups are depicted on schematics adapted from the rat brain atlas (Paxinos and Watson 1998).

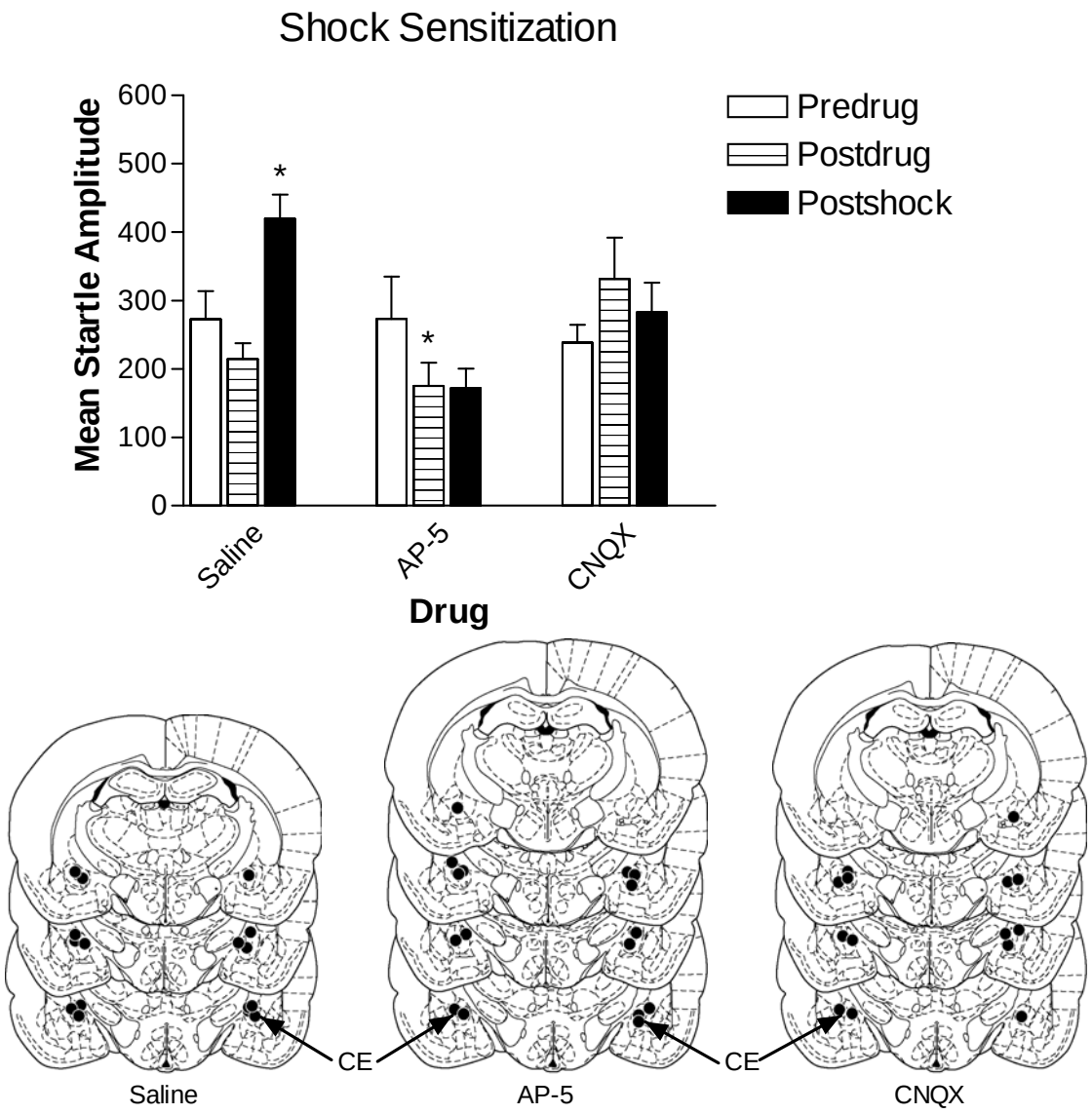

Figure 6.1: Effect of glutamate antagonists on shock sensitization in the central amygdala.

Mean (± S.E.M.) acoustic startle amplitudes after infusion of saline (N=8), 5µg AP-5 (N=8) or 5µg CNQX (N=7) into the central amygdala. Acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (post-drug) and after foot-shock administration (post-shock). * denotes a significant increase between post-drug ad post-shock reactivity in the saline group. Additionally, a significant attenuation in startle reactivity was found in the AP-5 group between pre-drug and post-drug responses, $P<0.05$.

Figure 6.1 illustrates that infusion of AP-5 and CNQX affected the shock augmented startle effect. Both groups failed to show a significant increase in startle responses after footshock. A 3 (drug) X 3 (test) repeated measures ANOVA showed a strong interaction ($F_{4,40}$=8.79, P<0.0000). As illustrated in Figure 6.1, infusions of AP-5 and CNQX blocked the shock sensitization effect as compared to the saline group. Simple effects analyses revealed that saline-infused rats exhibited no significant change between pre-drug and post-drug infusion (F=1.90, n.s.), but showed a significant increase in the acoustic response after foot-shock application, post-drug/post-shock (F=41.70, P<0.00000). Thus the cannulae placement and infusion procedure did not affect the immediate fear arousal produced by footshock.

As depicted in Figure 6.1, the infusion of AP5 significantly attenuated the ability to respond to noise bursts. Simple effects analyses between pre-drug/ post-drug showed a significant attenuating effect (F=5.49, P<0.03), and this could be interpreted as an ataxic response. Simple effects analyses between post-drug and post-shock were not significant (F=0.01, n.s.) thus indicating that rats failed to show shock sensitization. This effect could have been due to ataxia or to the inhibitory effects of AP-5 on immediate fear arousal. To further investigate this, a dose response study was conducted which will be investigated in the next experiment.

As illustrated in Figure 6.1, CNQX infusion enhanced post-drug startle but not post-shock startle. Simple effects analyses between pre-drug and post-drug produced a nearly significant increase (F= 4.32, P<0.051) suggesting that CNQX has an excitatory function, although this reaction was not augmented after footshock application. Simple effects analyses between post-drug and post-shock showed a non-significant effect

(F=2.05 n.s.). In fact, some attenuation of startle responses was evident. To eliminate a ceiling effect a t-test between saline post-shock and CNQX post-shock was performed. This yielded a significant difference between saline and CNQX (one tailed test, t(13)=2.50, P=0.01). Post-shock CNQX rats did not reach the same level of acoustic startle responses as the saline group, thus indicating that a ceiling effect was not a factor. Despite the fact that CNQX enhanced startle after infusion, this effect was not transferred to enhanced post-shock startle. The conclusion was that CNQX attenuated immediate fear arousal produced by footshock.

This results are at variance with results reported by Walker and Davis (1997b). Using a light-enhanced startle paradigm (an index of anxiety, which measured differences between noise bursts prior to bright light exposure and after), Walker and Davis (1997b) noticed that NBQX (a glutamate antagonist similar to CNQX) did not suppress light-enhanced startle. They concluded that the central amygdala was not involved in receiving initial information about the environment, but only in expressing information received from the basolateral amygdala.

An alternative explanation could be that non-NMDAr antagonism is involved in pain perception (Ikeda, Takahashi, Inoue, & Kato, 2007; Neugebauer, Li, Bird, Bhave, & Gereau, 2003). Since light enhanced startle does not involve pain, NBQX had no effect on the expression of anxiety. Conversely, fear expression produced by shock sensitization has an element of pain arising from footshock, thereby suggesting a possible reason for why CNQX blocked the response to footshock enhanced startle.

The inhibitory effects of AP-5 and CNQX on shock enhanced startle could also be due to a failure to perceive footshock. To investigate this, data collected 250 ms before

and 250 ms after footshock onset were compared. Figure 6.2 illustrates that rats infused with AP-5 and CNQX were not as vigorous in their shock reactivity compared with saline-infused rats.

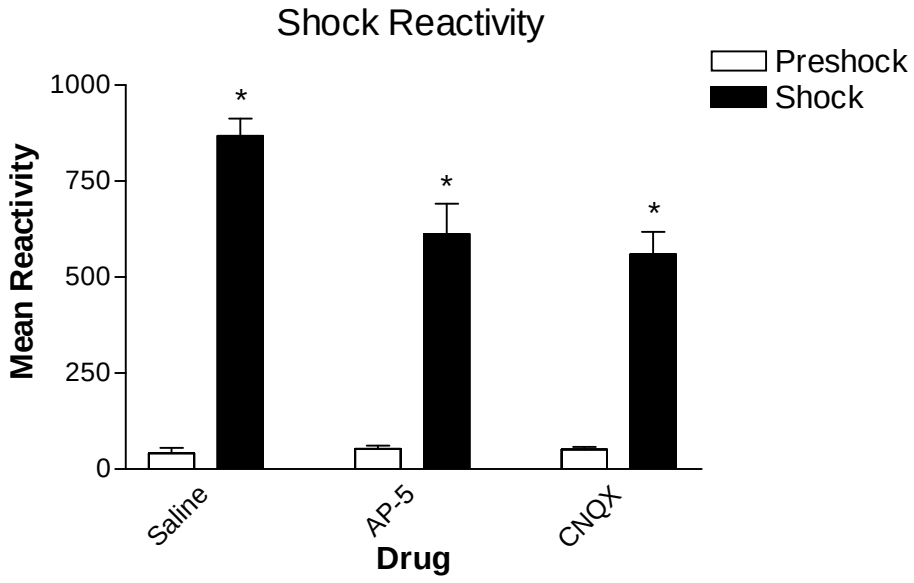

Figure 6.2: Effect of glutamate antagonists on shock reactivity.

Mean reactivity (± SEM) of saline, AP-5 and CNQX 250 ms before and 250 ms during footshock application. * denotes that all three groups showed significant increases between pre-shock and shock reactivity levels, $p<0.05$.

Figure 6.2 outlines the effect of drug infusion on foot-shock associated movement measured 250 ms before and 250 ms during footshock. A 3 (drug) X 2 (test) repeated measures ANOVA produced a significant interaction ($F_{2,20}=7.77$, $P<0.003$), a significant main drug effect ($F_{2,20}=5.85$, $P<0.01$) and a significant main shock effect ($F_{1,20}=314.51$, $P<0.0000$). Simple effects analyses showed significant differences between saline and AP-5 and saline and CNQX ($F=7.16$, $P<0.01$, $F=9.97$, $P<0.005$) respectively. All groups showed significant effects of foot-shock but the reactivity of the AP-5 and CNQX groups were somewhat attenuated compared with saline infused rats.

The transfer of drugs to other amygdaloid areas could not be completely excluded. However, the results in Section 2 showed that both CNQX and AP-5 failed to suppress the shock sensitization effect in the basolateral amygdala. The results reported in this experiment indicated that the drugs did not flow towards the basolateral amygdala and it is reasonable to suggest that drugs did not diffuse to other surrounding areas.

Overall these results indicate that glutamate antagonists reduce immediate fear arousal. However, it could not be discounted that this effect was due to a form of ataxia. To investigate this, a dose-response study of AP-5 was undertaken.

Experiment 18: Dose-response study of AP-5 during fear arousal produced by footshock.

An important finding was that antagonising the glutamate receptors could suppress the shock sensitization effect in the central amygdala. However, it could be argued that infusion of AP-5 produced ataxia because of the attenuated responses after infusion and thus inhibited the ability to respond to noise after infusion and that this was the reason for the attenuated acoustic startle responses after foot-shock presentation. And indeed the dose was relatively high, namely 5µg/ µl. To further investigate this potential ataxic consequence, effects of five doses of AP-5 were assessed. Sixty rats were surgically prepared and infused with AP-5 concentrations ranging from 5µg/µl to 0.3µg/µl and compared with a new saline control group. Sixteen rats were rejected after histological verification. Some of the rejected rats had cannulae on the border of or into the basolateral amygdala. These data points were not included in the analyses.

Figure 6.3 shows the mean startle amplitude of rats under influence of 6 different AP-5 concentrations during the shock sensitization paradigm. Cannulae placements in the

central amygdala of both groups are depicted on schematics adapted from the rat brain atlas (Paxinos and Watson 1998).

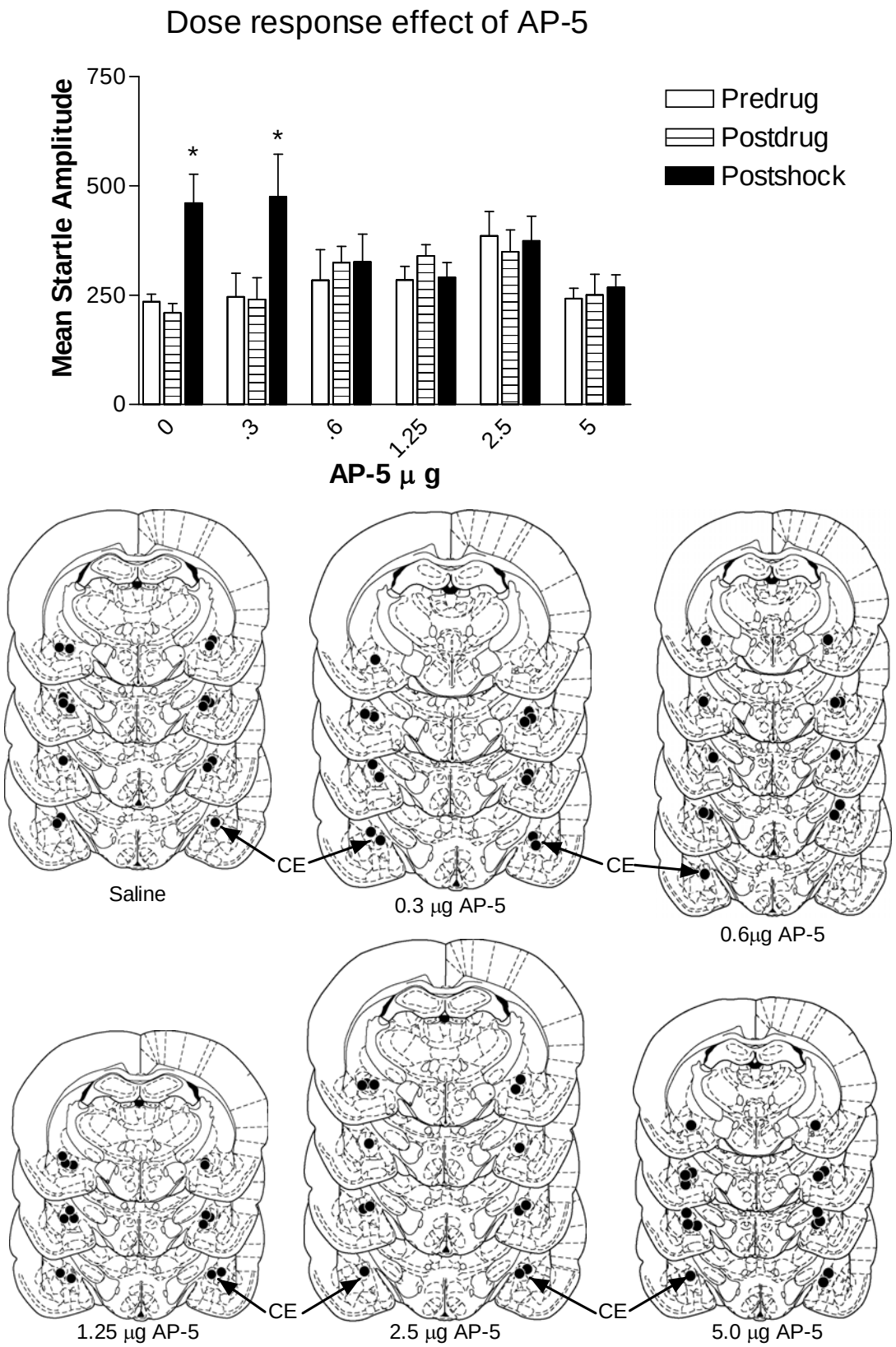
Dose response effect of AP-5
Mean Startle Amplitude
750
500
250
0
Predrug
Postdrug
Postshock
*
*
0
.3
.6
1.25
2.5
5
AP-5 μ g
CE
CE
Saline
0.3 μg AP-5
0.6μg AP-5
CE
CE
1.25 μg AP-5
2.5 μg AP-5
5.0 μg AP-5

Figure 6.3: Dose response effect of AP-5 during shock sensitization.

Mean (± S.E.M.) acoustic startle amplitudes as a function of AP-5 infusion into the central amygdala. Acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (postdrug) and after foot-shock administration (post-shock). Rats infused with saline (N=8) or 0.3 µg AP-5 (N=7) showed a sensitization effect to noise after footshock presentation, * $P<0.05$. Rats infused with 0.6µg (n=6), 1.25µg (N=8), 2.5µg (N=7) and 5µg AP-5 (N=8) all reliably attenuated their acoustic startle response after foot-shock presentation.

Figure 6.3 illustrates that drug concentrations ranging from 5µg/µl down to 0.6µg/µl successfully prevented the expression of shock augmented startle. The smallest effective dose rate was 0.6 µg AP-5 and this was similar to an effective dose used by Jasnow, Cooper and Huhman (2004) in their investigation of conditioned defeat. A 3 (test) x 6 (drug) repeated measures ANOVA revealed a significant interaction and main test effect ($F_{10,79}$=3.09, $P<0.002$) and ($F_{2,79}$= 8.33, $P<0.0005$) respectively. Simple effects analyses of the pre-drug/post-drug data showed that none of the groups altered their behaviour after drug infusion compared with pre-drug infusion, saline (F=0.38), 0.3µg (F=0.01), 0.6µg (F=0.7), 1.25µg (F=1.83), 2.5µg (F=0.71), and 5µg (F=0.04). Analyses of the post-drug/post-shock simple effects revealed that rats infused with doses of AP-5 from 5 µg down to 0.6µg were significantly impaired in foot-shock-enhanced fear expression, 5µg (F=0.07), 2.5µg (F=0.13), 1.25µg (F=0.57) and 0.6µg (F=0.001). However, rats in the saline and the 0.3µg AP-5 group significantly amplified their acoustic startle response after foot-shock application (F=15.46, $P<0.0003$) and (F=11.83, $P<0.001$) respectively. Thus, the lowest effective dose of AP-5 that prevented the shock sensitization effect was 0.6 µg/µl.

To determine if the effect was due to a decrease in footshock perception, data collected 250 ms before and during footshock were compared. Figure 6.4 illustrates that all groups were able to respond with vigour to foot-shock application.

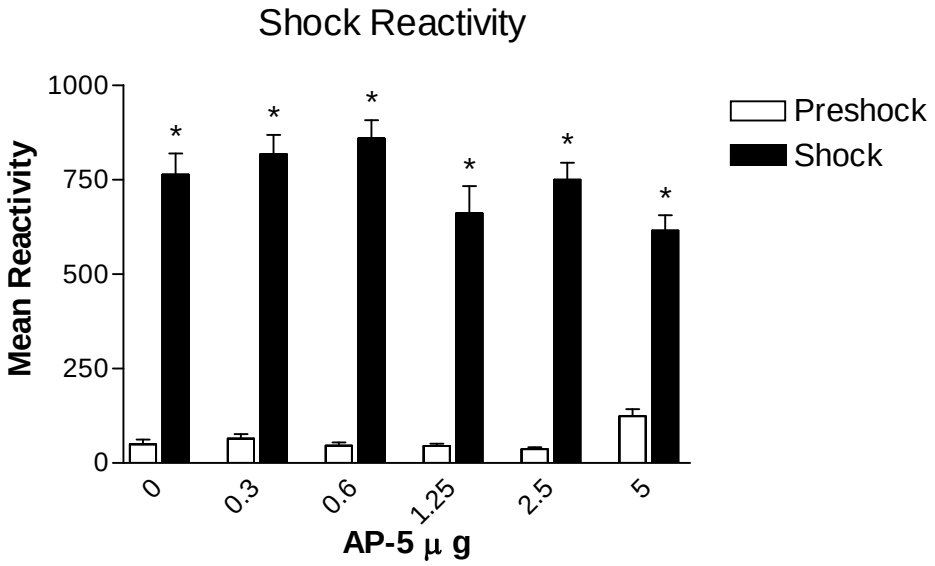

Figure 6.4: Dose response effect of AP-5 on shock reactivity.

Mean reactivity (± SEM) of saline, and 6 AP-5 drug concentrations 250 ms before and 250 ms during footshock application. * denotes that all groups showed significant increases between pre-shock and shock reactivity levels, $p<0.05$.

A 2 test (pre-shock/shock) x 6 (drug) repeated measures ANOVA showed a significant interaction and main shock effect ($F_{5,38}=4.49$, $P<0.002$) and ($F_{1,38}=944.0$, $P<0.00000$) respectively. As depicted in Figure 6.4, all groups exhibited significant movement amplitudes, indicating that footshock reactivity was not significantly influenced by AP-5 infusion. Even though the 1.25 group showed a slightly attenuated effect this was not significantly different from the saline group. This was established by simple effects analyses between saline and 1.25 µg/µl ($F=1.95$, n.s.). The 5 µg/µl AP-5 group reached a near significant difference between saline and AP-5 ($F=4.06$, $p=0.051$

n.s.). This result was similar to that presented in Figure 6.2 indicating that a high dose of AP-5 can reduce overall shock reactivity.

The findings that glutamate suppresses footshock-enhanced immediate fear arousal in the shock sensitization paradigm suggests two possible interpretations namely (1) does the shock sensitization paradigm elicit a central fear state in the central amygdala that is suppressed by glutamate, or (2) is the central amygdala involved in pain perception as postulated by Neugebauer and Li (2002)? It is unlikely that the central fear state itself is affected because the results reported in Sections 2 and 3 indicate that the central fear state is at least located in the basolateral amygdala. Therefore, glutamate neurotransmission may prevent pain which in turn affects conditioning to aversive stimuli (Goosens & Maren, 2003). Results reported by Shors and Mathew (1998) suggest that the infusion of AP-5 before footshock-elicited stress had no effect on acquisition of a conditioning task 24 h later. Conversely, AP-5 infusion into the basolateral did have an effect. This was interpreted as a NMDA-induced learning deficit in the basolateral amygdala. The failure for AP-5 to affect learning in the central amygdala could have been due to AP-5 suppressing pain, and because of its non-associative and temporary effect, it did not affect stress facilitated fear acquisition 24 h later.

From the shock data presented in Figure 6.4 it is clear that the animals perceived shock but as Borszsz (1993, 1995) states, the physical properties of shock consist of both reflexes and pain. The reactivity data most likely reflects the reflex but not necessarily the pain. If this is true then the suppression of pain prevents the expression of fear arousal and thus fear conditioning. This also suggests that pain is a component of fear conditioning, but pain should not be necessary for fear expression at a later date. It was

likely that infusion of glutamate prior to fear-potentiated startle would not suppress fear expression because conditioned fear expression does not entail pain during fear recall. Whether or not this was the case will become clear in Section 5.

Experiment 19: The role of GABA neurotransmission in fear arousal produced by footshock.

Major inhibitory neurotransmission is mediated by $GABA_a$ receptors not only in the basolateral but also in the central amygdala (Delaney & Sah, 1999; Sun, Yi, & Cassell, 1993). Functional inactivation using large doses of muscimol prevented acquisition of freezing behaviour (Holahan & White, 2004; Wilensky, Schafe, Kristensen, & LeDoux, 2006) and delayed conditioning of membrane responses (Burhans & Schreurs, 2008). More importantly, functional inactivation reduced freezing behaviour during footshock to a comparable level to rats that received no footshock (Holahan & White, 2004). This indicated a function for the central amygdala and a potential role for GABA in fear processing. The question asked was, is a GABAergic neural mechanism involved in immediate fear arousal produced by foot-shock? The $GABA_a$ agonist, muscimol, blocked the shock-augmented startle effect in the basolateral amygdala. Would the same effect be found in the central amygdala? To that end, the identical concentration of 0.005 μg/μl muscimol that successfully prevented shock sensitization in the basolateral amygdala was infused into the central amygdala

Ten rats had bilateral cannulae implanted aiming for the central amygdala. Of those, 3 were excluded from statistical analyses after histological verification. Rats with cannulae outside of or on the border between the central and basolateral amygdala were

excluded. A total of 7 rats in the muscimol group remained. These were compared to the saline group of Experiment 17.

Startle data of rats infused with either saline or 0.005µg/µl muscimol during the shock sensitization paradigm are presented in Figure 6.5. Pre-drug/post-drug/post-shock startle responses were compared, in order to assess the effect of muscimol on startle responses before and after footshock. Furthermore, the effect of muscimol on footshock reactivity was evaluated. Cannulae placements in the central amygdala of both groups are depicted on schematics adapted from the rat brain atlas (Paxinos & Watson, 1998).

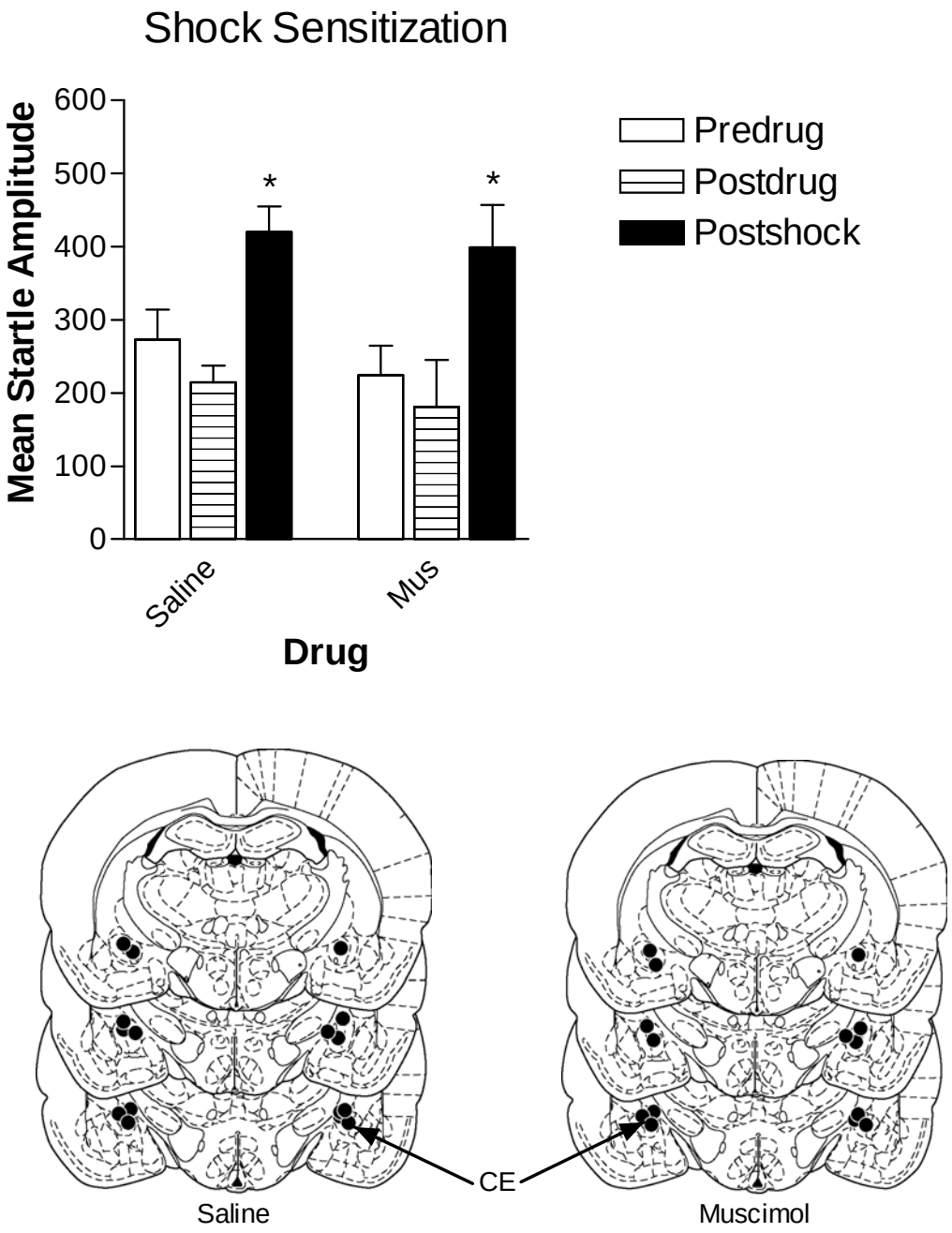

Figure 6.5: Effect of muscimol on shock sensitization in the central amygdala.

Mean (± S.E.M.) acoustic startle amplitudes after infusion of saline or 0.005µg/µl muscimol, into the central amygdala during the shock sensitization paradigm. Rats infused with saline (N=8), muscimol (N=7), showed a significant increase in post-shock startle relative to post-drug startle, * $P<0.05$. CE, Central amygdala.

Figure 6.5 illustrates that both muscimol and saline infusion failed to affect shock sensitization in the central amygdala. Both groups showed significant startle increases between post-drug and post-shock. A 2 (drug) X 3 (test) repeated measures ANOVA revealed a highly significant test effect ($F_{2,26}$=17.92, P<0.0000). Simple effects analyses showed no significant differences between the pre-drug and post-drug tests. Thus, the infusion of the drugs did not affect startle reactivity. But all groups showed a significant increase in responses between the post-drug/post-shock test, saline (F=29.7, P<0.000), and muscimol (F=29.3, P<0.0000) thus indicating that footshock significantly augmented startle amplitudes, and that muscimol failed to prevent this. The data analyses and Figure 6.5 clearly showed that $GABA_a$ neurotransmission was not involved in immediate fear arousal during the shock sensitization paradigm in the central amygdala. However, Sanders and Shekhar (1995) reported that the infusion of a small dose of muscimol attenuated anxiety in a social interaction test. This difference is likely because anxiety and fear are regulated via different amgygdalar neural mechanisms. For example, fear expression is a simpler, less cognitively laden process than the expression of anxiety (McNaughton & Corr, 2004). Thus a role for GABA neurotransmission cannot be ruled out but it appears not be involved in the central fear state as shown by the results of the shock sensitization paradigm.

A GABA neural mechanism was not involved in the perception of foot-shock either. Figure 6.6 showed the reactivity responses measured 250 ms before and during foot-shock application while under the drug's influence. Both groups showed significant increases between pre-shock and during footshock. A 2 (drug) X 2 (test) repeated

measures ANOVA yielded a highly significant main effect for shock ($F_{1,31}$=504.06, P<0.000000).

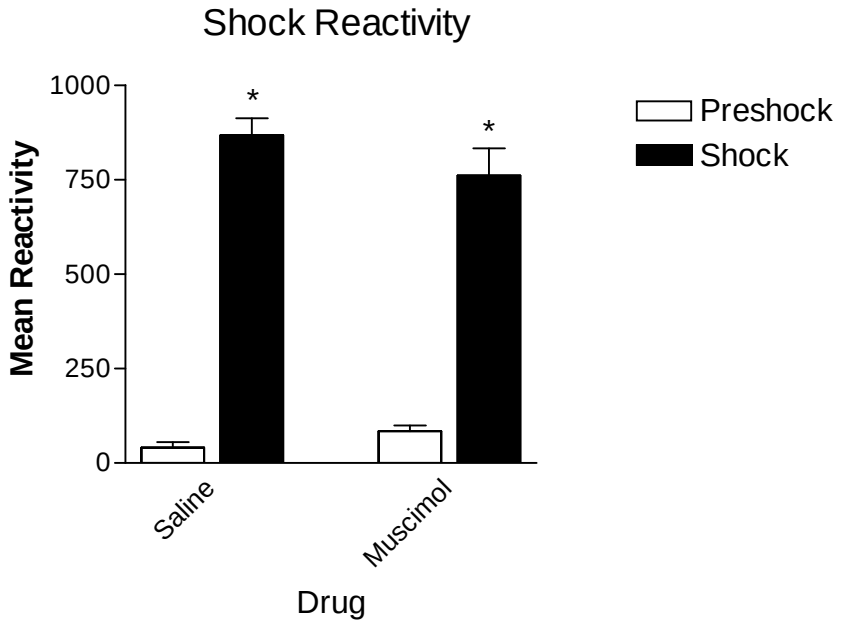

Figure 6.6: Effect of muscimol on shock reactivity in the central amygdala.

Mean reactivity (± SEM) of saline and muscimol 250 ms before and 250 ms during footshock application. * denotes that both groups showed significant increases between pre-shock and shock reactivity levels, p<0.05.

As is evident from Figure 6.6, muscimol did not affect responses to footshock. The lack of an effect by muscimol on foot-shock was also noted by Wilensky, et al (2006) who used a high dose of muscimol to inactivate the central amygdala during three rapid foot-shock presentations.

The findings that high concentrations of muscimol prevent fear acquisition have previously been explained as a mnemonic effect (Holahan & White, 2004; Wilensky, Schafe, Kristensen, & LeDoux, 2006). However, the inhibitory effects on acquisition may

have an alternative explanation. Inactivation may have reduced footshock-elicited pain and this prevented fear acquisition, because lesions of the central amygdala before conditioning using non-painful US stimuli do not prevent conditioning (Hatfield, Graham, & Gallagher, 1992). In addition, inactivation using tetrodoxin had no effect on the acquisition of cocaine-paired associative learning (Kruzich & See, 2001), thereby suggesting that perhaps inactivation of the central amygdala during a painful US presentation prevents US-CS acquisition but not during a non-painful US-CS presentations.

Even though functional inactivation appears to prevent fear acquisition, it is unlikely that a GABAergic system is involved in the expression of footshock-enhanced acoustic startle in this nucleus.

Experiment 20: The role of protein synthesis inhibition in fear arousal produced by footshock.

The central amygdala has been shown to support long term potentiation (Sandkuhler, 2007). De novo protein synthesis is required to elicit neural changes associated with long-term potentation (de Armentia & Sah, 2007) and inhibition of protein synthesis has been shown to prevent conditioned taste acquisition (Bahar, Samuel, Hazvi, & Dudai, 2003). As experiment 17 showed both CNQX and AP-5 prevented fear arousal but not footshock perception, thereby suggesting that involvement of a form of long-term potentiation cannot be excluded. To further eliminate long term potentiation as the mechanism responsible for fear suppression produced by footshock, 10 rats were surgically prepared and infused with anisomycin prior to footshock presentation. Histological verification eliminated 3 rats, leaving 7 data points, these were

compared to the results of the saline group reported in Experiment 17. The results are shown in Figure 6.7 which demonstrates that rats infused with anisomycin exhibited enhanced sensori-motor activity under the drug's influence. This enhancement was not significantly increased after footshock stimulation. The post-shock responses were similar to the saline group, indicating that infusion of anisomycin did not significantly affect immediate fear arousal produced by footshock.

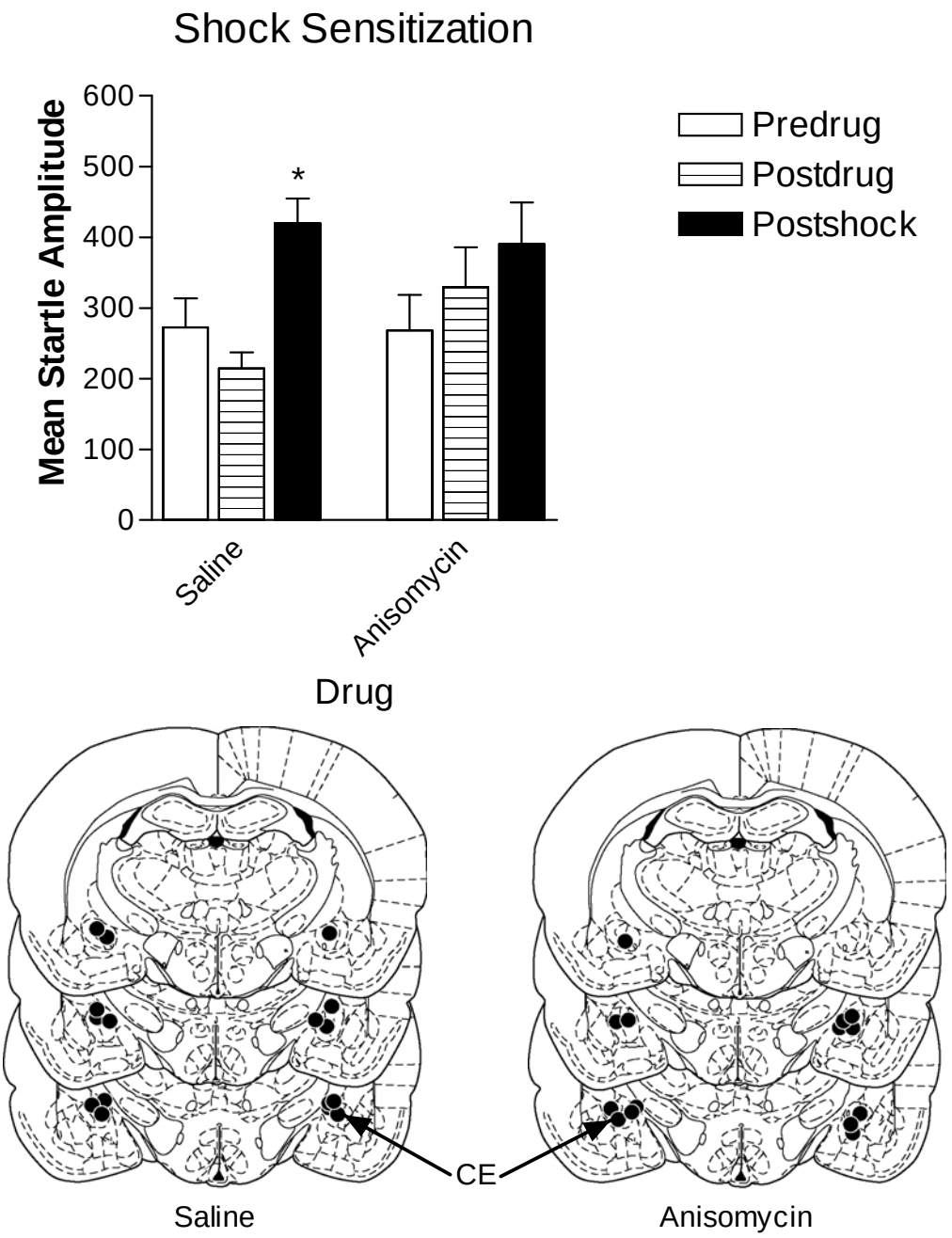

Figure 6.7: Effect of anisomycin on shock sensitization in the central amygdala.

Mean (± S.E.M.) acoustic startle amplitudes after infusion of saline or 80µg anisomycin into the central amygdala. Acoustic startle amplitudes were measured prior to drug infusion (pre-drug), after drug infusion (postdrug) and after foot-shock administration (post-shock). Rats infused with saline (N=8) showed a significant sensitization effect. * $P<0.05$. Anisomycin (N=7) affected startle reactivity which masked a significant effect.

A 2 (drug) X 2 (test) repeated measures ANOVA yielded a significant test effect ($F_{2,24}$=12.31, P<0.0002) and a near significant interaction ($F_{2,24}$=3.28, P<0.055). Simple effects analyses revealed that saline-infused rats were not affected (F=2.85, n.s.) and could clearly display increased startle after footshock (F=18.17, p= 0.001). Conversely, the anisomycin group showed insignificant augmented startle after infusion (F=2.42, n.s.). This augmentation was further enhanced after footshock stimulation but did not reach a statistically significant difference (F=2.10, n.s.). The lack of significance can be interpreted as a failure to suppress fear arousal, because the post-shock level was similar to the saline group. This was further analysed by completing an independent t-test between saline and anisomycin post-shock data (t(13)=1.77, p=0.33, n.s., one tailed). These results show that both groups had comparable augmented startle after footshock application. But this was masked by augmented startle activity during the post-drug testing. The augmentation in post infusion startle was also noted during the fear potentiated startle paradigm (see Section 5, Experiment 24). Furthermore, this effect was absent when anisomycin was infused into the basolateral amygdala.

This increased reactivity was not seen during the 250 ms pre-shock data depicted in Figure 6.8 thereby suggesting that it was not a general non-specific effect of the drug but may have arisen from a small increase in post-drug anxiety. Figure 6.8 illustrates data collected during the 250 ms pre-shock activity and is compared to 250 ms shock reactivity.

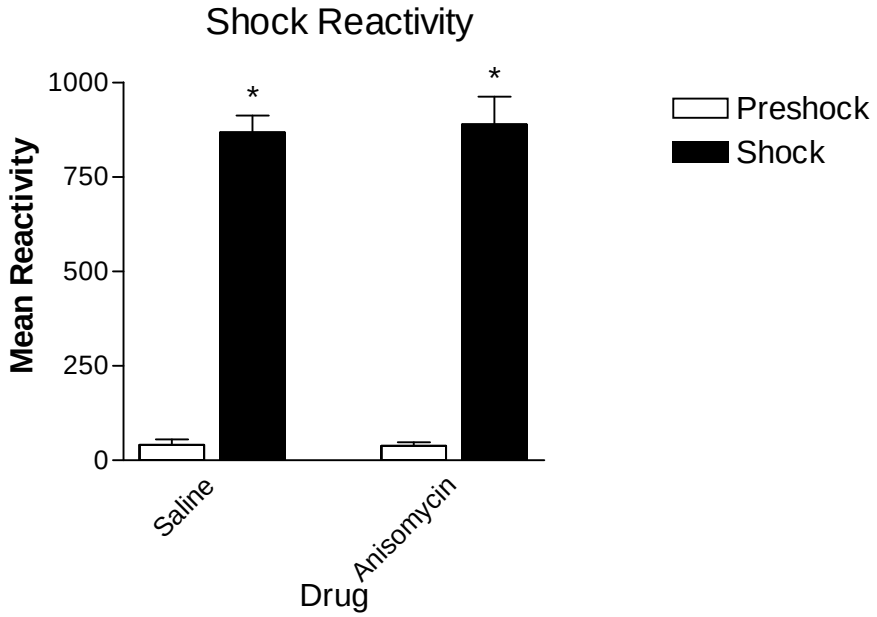

Figure 6.8: Effect of anisomycin on shock reactivity in the central amygdala.

Mean reactivity (± SEM) of saline and anisomycin 250 ms before and 250 ms during footshock application. * denotes that both groups showed significant increases between pre-shock and shock reactivity levels, $p<0.05$.

Analyses of footshock reactivity revealed that behaviour under the influence of anisomycin was very similar to saline, including pre-shock levels. A 2 (drug) X 2 (test) repeated measures ANOVA yielded a highly significant effect for shock ($F_{1,12}$= 332.06, P=0.00000), thus indicating that footshock was perceived and reacted to in a manner similar to the control group.

While the fact that anisomycin failed to suppress the shock sensitization effect was not unexpected, it had to be investigated to eliminate any long-term potentiation effects. This result strengthens the argument that suppression of shock sensitization via glutamate antagonism was not the result of inhibition of long term potentiation processes, but a pain perception deficit.

Experiment 21: The role of dopamine agonists in fear arousal produced by footshock.

The ventral tegmental area and the central amygdala have a reciprocal relationship (Gelowitz & Kokkinidis, 1999) involving a dopaminergic mechanism that controls motor activation and attention (Gallagher & Holland, 1994). The central amygdala also modulates internal and external cues via dopamine populations involving the sympathetic nervous system and attentional mechanisms (Fudge & Emiliano, 2003). For example, both D1 agonists and antagonists could prevent the expression of morphine-induced conditioned place preference, suggesting a role for dopamine in cognitive functioning (Zarrindast, Rezayof, Sahraei, Haeri-Rohani, & Rassouli, 2003). The acquisition of conditioned avoidance responses was also affected by activation of dopamine receptors (Reis, Masson, De Oliveira, & Brandao, 2004). A D1 agonist infused prior to fear conditioning has been shown to facilitate freezing behaviour during later fear expression tests (Guarraci, Frohardt, & Kapp, 1999). Additionally, dopamine has been shown to alter attention to salient cues specific to mnemonic processes (Wise, 2004). Thus a potential role for a dopaminergic system in the central amygdala during shock sensitization could not be ruled out.

Twenty four rats were bilaterally cannualized aiming for the central amygdala. After histology 8 rats that had received 3µg/µl quinpirole and 12 rats from the 4µg/µl SKF 38393 group with correctly placed cannulae were included in the statistical analyses. These two groups were compared to the saline control group from Experiment 17. Figure 6.9 shows the comparison between saline, SKF 38393 and quinpirole before and after

drug infusion. Cannulae placements in the central amygdala of all groups are depicted on schematics adapted from the rat brain atlas (Paxinos and Watson 1998).

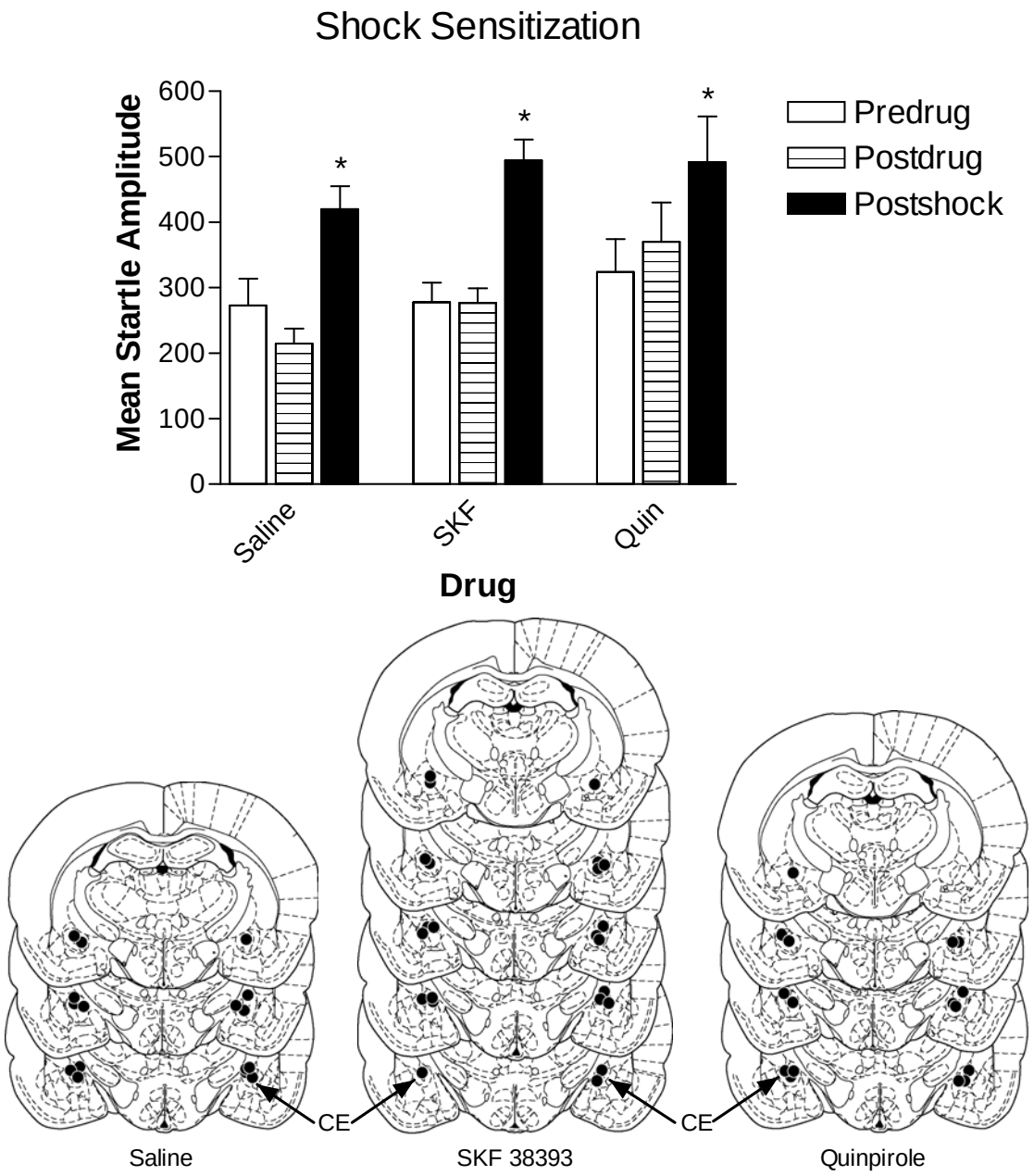

Figure 6.9: Effect of dopamine agonists on shock sensitization in the central amygdala.

Mean (± S.E.M.) acoustic startle amplitudes after infusion of 4.0µg/µl SKF 38393 or 3.0 µg/µl quinpirole, into the central amygdala during the shock sensitization paradigm. Rats infused with saline (N=8), SKF 38393 (N=12) and quinpirole (N=8) showed a significant increase in post-shock startle relative to postdrug startle, * $P<0.05$.

As can be clearly seen in Figure 6.9, neither dopamine agonist affected the shock sensitization effect. The groups showed significant differences between post-drug and post-shock acoustic startle responses. A 3 (drug) X 2 (test) repeated measures ANOVA yielded a highly significant main test effect ($F_{2,50}$=504.06, P<0.000000). Simple effects analyses showed no effect of the drug on acoustic startle responses, but a significant increase in acoustic startle responses was noted after footshock, saline (F= 24.74, P=0.0000), SKF 38393 (F=41.86, P=0.0000) and Quinpirole (F=8.63, P=0.007).

Footshock was not significantly affected by the dopamine agonists. Figure 6.10 illustrates the reactivity measured 250 ms before and 250 ms during footshock.

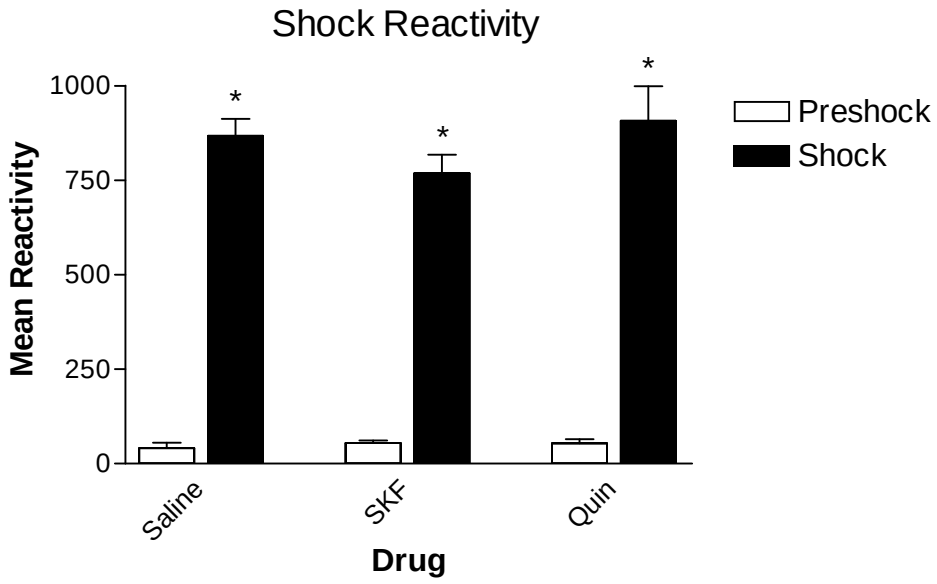

Figure 6.10: Effect of dopamine agonists on shock reactivity in the central amygdala.

Mean reactivity (± SEM) of saline SKF 38393 and quinpirole 250 ms before and 250 ms during footshock application. * denotes that all groups showed significant increases between pre-shock and shock reactivity levels, p<0.05.

Neither the D1 agonist SKF 38393 nor the D2 agonist quinpirole affected footshock reactivity - all groups showed significant reactivity during footshock as compared to

before footshock. Statistical analyses confirmed the results shown in Figure 6.10. A 3 (drug) X 2 (test) repeated measures ANOVA revealed a highly significant main test effect ($F_{1,25}$=441.89, P<0.000000).

Overall, dopamine agonists had no measurable effect on either fear arousal or on footshock reactivity, indicating that dopaminergic systems located in the central amygdala do not affect immediate fear arousal produced by footshock. The shock sensitization paradigm does not appear to test for attention to salient cues associated with fear conditioning. To totally exclude dopaminergic effects this paradigm should be repeated with both D1 and D2 antagonists because of the possible modulating effects of dopamine (Zarrindast, Rezayof, Sahraei, Haeri-Rohani, & Rassouli, 2003).

Summary of results

The main finding in this section (and as shown in Experiments 17 and 18) was that a glutamatergic system is involved in immediate fear arousal produced by footshock. Both NMDA and non-NMDA receptors appear to be involved, because AP-5 and CNQX were able to block the shock sensitization effect. This effect has been interpreted as a reduction in pain perception.

In Experiment 19 it was shown that the GABAergic system was not involved in the shock sensitization effect.

Experiment 20 showed that the novo protein synthesis, necessary for LTP, was not required during footshock-enhanced fear expression. This suggests that the shock sensitization effect was not a form of rapid fear acquisition.

Lastly, Experiment 21 demonstrated that neither of the dopamine agonists affected fear expression. Thus, attention to context or other salient cues was probably not required during the shock sensitization paradigm.

RESULTS SECTION 5: Experiments 22-27.

The role of glutamate, protein synthesis, GABA, and dopamine in the expression of fear potentiated startle in the central amygdala.

As was discussed earlier, footshock comprises three components namely, reflex, pain and emotion (Borszcz, 1993, 1995). By comparing the results of the shock sensitization paradigm to the fear potentiated startle paradigm it is possible to evaluate overlaps between an immediate fear state and a conditioned fear state. It was demonstrated that the basolateral amygdala processes the emotive component of footshock induced fear arousal. Immediate fear arousal was suppressed by enhancing the inhibitory effects of the GABAergic system. A dose response study clearly showed that a low concentration of muscimol could inhibit immediate fear arousal produced by footshock. Moreover, a low dose of muscimol also prevented fear expression to a CS. These results were interpreted as GABA being a mechanism involved in processing the emotive component produced by footshock in the basolateral amygdala.

In Section 4 it was demonstrated that a GABAergic mechanism in the central amygdala was not involved in immediate fear arousal produced by footshock. But, glutamate antagonism by either AP-5 or CNQX infusion abolished the shock sensitization effect in the central amygdala. A dose response study clearly demonstrated a significant role for AP-5 in suppressing immediate fear arousal. This was interpreted as suppression of footshock-produced pain responses.

The next question was: what role does glutamate play in the expression of fear-potentiated startle in the central amygdala? If glutamate is involved in pain suppression (Bennett, 2000; Fu et al., 2008) then neither AP-5 nor CNQX should suppress Conditioned fear expression, because recall and the expression of fear to a CS is unlikely to include physical pain. Conversely, if the central amygdala, like the basolateral amygdala is also involved in processing the fear component of foot-shock, as opposed to processing pain then, it would be expected that both AP-5 and CNQX would suppress fear-potentiated startle.

To determine if the central amygdala was also involved in fear expression rats were treated with the same drugs namely, AP-5, CNXQ, muscimol, anisomycin, SKF 38393 and quinpirole, and submitted to the fear potentiated paradigm. The drugs and paradigm were identical to those used in Section 3.

A summary of the fear potentiated startle paradigm is as follows: rats received 30 conditioning trials and were exposed to 4 manipulations: (1) A short fear test to show rats experienced fear prior to the main fear test (2) a pre-drug/post-drug test to show the effect of drug on acoustic startle reactivity (3) the main fear test to show the effect of the drug on fear potentiated startle expression (4) a '24 hour later' test to show no long-term detrimental effects of the drug itself and the previous manipulations on fear expression.

It was expected that all groups would show significant fear during the short test, no effect of drug on acoustic startle responses and, depending on the drug, either a failure to express fear or not during the main fear test. During the final 24-hours-later test it was expected that the rats would still exhibit significant fear. All drugs were compared to the

same saline group. Again the same drugs that were used for Sections 2, 3 and 4 were investigated in this last section.

Experiment 22: The role of NMDA antagonism in fear expression during the fear potentiated startle paradigm.

The first experiment in this section investigated NMDA neurotransmission during fear expression to CS presentation. As shown during the shock sensitization paradigm, both the NMDA antagonist AP-5 and the non-NMDA antagonist CNQX could block immediate fear arousal produced by foot-shock. As asked previously, was this due to the suppression of a central fear state or due to a failure in pain perception? If it was pain perception than it would be expected that neither AP-5 nor CNQX would block fear expression during the main fear test. On the other hand, if it was suppression of the central fear state, then both AP-5 and CNQX should block fear expression during the main fear test, similar to the GABAergic effect demonstrated in the basolateral amygdala.

To investigate this question rats were infused with AP-5 (5µg/µl) prior to fear testing and compared to the control saline group. For this experiment, 20 rats were surgically prepared and after histological verification 3 rats were excluded. Figure 7.1 depicts the effect of AP-5 in conditioned fear expression, whereby AP-5 has no obvious effect on startle responses after CS presentation during the main fear test.

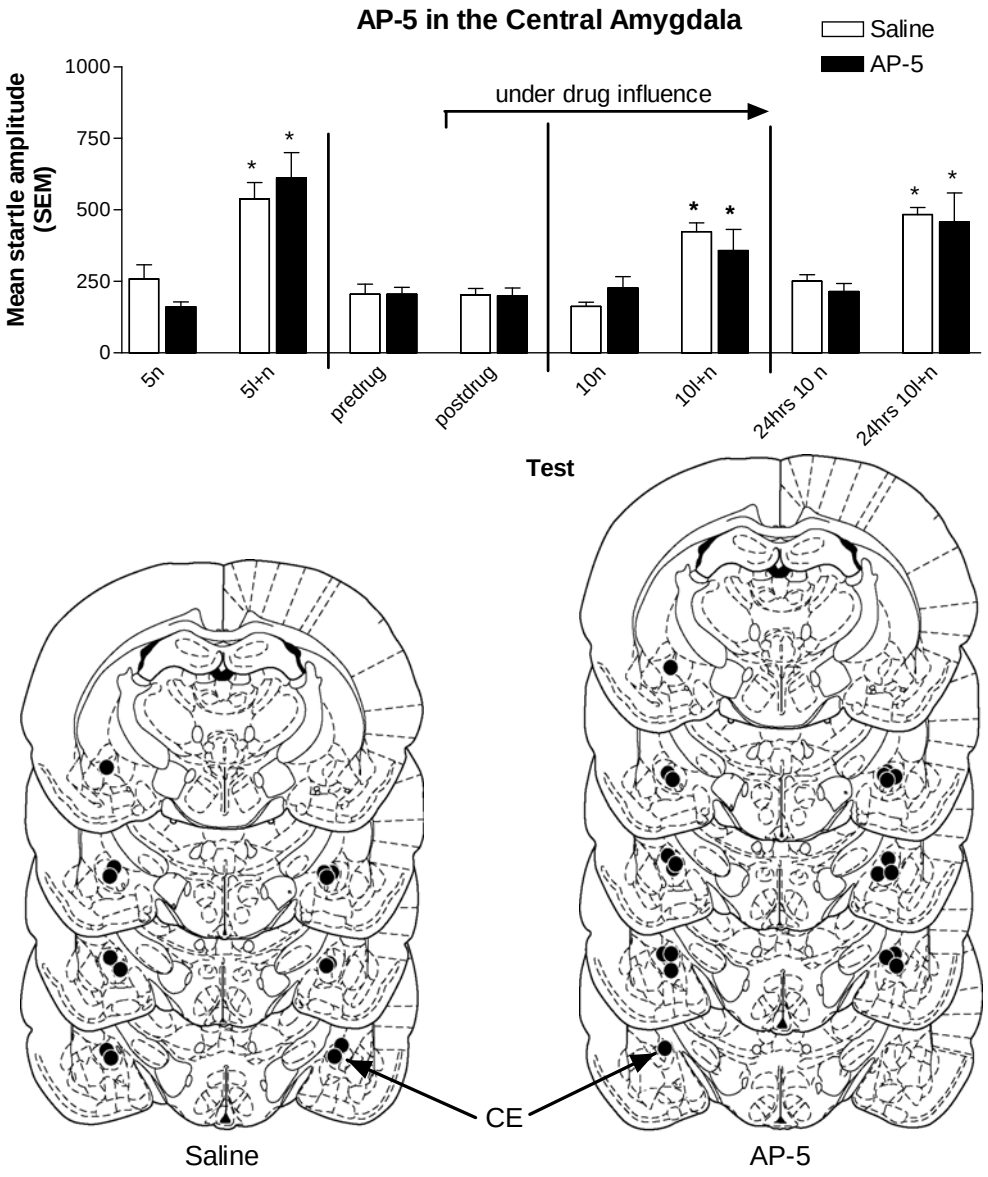

Figure 7.1: Effect of AP-5 in fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a predrug /postdrug test (20n, 20n) where rats were infused with saline (N=7) or 5µg AP-5 (N=10) after the predrug test. This was followed by a fear expression test under drug influence (10n 10l+n), and 24 h later with another fear test (24h 10n/24h 10l+n). Rats infused with AP-5 were unaffected in their ability to display fear expression during the 10 noise 10 l+n test. * indicates significant increases in acoustic startle responses after CS presentation for each manipulation within each drug group, $P<0.05$. Cannulae placements in the amygdala are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

Figure 7.1 illustrates that AP-5 did not significantly affect fear expression as measured by a change in startle responses during the drugged 10 noise, 10 Light+ noise test. Statistical analyses using a 2 (drug) x 2 (test) repeated measures ANOVA yielded a significant main test effect ($F_{1,15}$=32.15, P<0.00004). Simple effects analyses showed that both saline and AP-5 groups experienced significant fear (F=24.20, P<0.0001, F=8.73, P<0.009 respectively). This lack of effect could have been due to the AP-5 drug concentration, but this is unlikely, because the same AP-5 (5µg/µl) drug concentration was very effective in blocking a central fear state during the shock sensitization paradigm in the central amygdala. This indicates that glutamate receptors are active during US processing, but that they were not required during CS processing. Moreover, this drug concentration was effective when infused into the basolateral amygdala during the fear potentiated startle paradigm. Thus, the lack of a drug effect was unlikely to be related to drug concentration or to the paradigm.

Twenty-four hours later, both groups still showed a significant fear effect. A 2 (drug) x 2 (test) repeated measures ANOVA yielded a significant main test effect ($F_{1,15}$=14.61, P<0.001) indicating that the procedures and the infusion of AP-5 had no lingering effects on the ability to express learned fear. The pre-drug/post-drug test, designed to show that AP-5 did not change the capacity to respond to noise during the fear test, also showed no significant effect. A 2 (drug) x 2 (test) repeated measures ANOVA failed to reveal a significant outcome (F=0.007, n.s.) thus indicating that AP-5 did not affect acoustic startle responses.

Furthermore, both groups experienced statistically significant fear during the short fear test. A 2 (drug) x 2 (test) repeated measures ANOVA showed a significant main

effect for test ($F_{1,15}$=35.57, P<0.00002). Overall the results indicate that both the control and AP-5 groups experienced fear prior to drug infusion, under drug influence, and twenty-four hours later.

This interesting finding that AP-5 failed to block conditioned fear expression but did suppress the expression of a central fear state produced by rapid footshock presentations invite some novel interpretations. In 2003 Goosen and Maren reported that the infusion of APV (similar to AP-5) into the central amygdala blocked conditioning of freezing behaviour to explicit auditory and contextual cues. They suggested this was due to a memory formation deficit. An alternative explanation could be that AP-5 blocked the ability of foot-shock to produce a central fear state via a reduction in pain perception and this could explain the association deficit. By not perceiving the footshock-induced pain, no significant association was formed. This also means that blocking pain perception is sufficient to prevent fear conditioning. Since both the basolateral and central amygdala receive information about the US and CS (Pare, Quirk, & LeDoux, 2004), this has implications for the 'exclusive' role of the basolateral region in the formation of CS/US associations. Research conducted by Goosens and Maren (2003) showed that residual information was stored after inactivation of the central amygdala post AP-5 infusion. Central amygdala- cannulated rats showed improved learning as opposed to basolateral-cannulated subjects, these rats showed no residual memory. Together these results may imply an order of effectiveness, namely blocking fear in the basolateral amygdala prevents fear conditioning, but blocking pain in the central amygdala also prevents conditioning but to a lesser extent. Thus blocking of pain is less effective than blocking of fear during conditioning.

However, once the US-CS association is formed then AP-5 has no influence on its expression. This raised the question of what exactly does AP-5 do during the US presentation and not do during CS presentation? The main difference between these two paradigms is pain perception, which is present during US and absent during CS presentation.

The strength and duration of the footshock used in this thesis is appropriate for showing the effect of pain since the associative effects produced by footshock depend upon the strength and duration of the footshock. Moderate footshocks of short duration (0.6 mA 0.5 s) are painful and very effective in generating fear responses that are easily paired with a CS to produce effective fear conditioning (Meagher et al., 2001). Conversely, severe shock (> 1mA), presented for longer durations (>2seconds) attenuates pain and impairs learning (Meagher et al., 2001). The research conducted in this thesis used a moderate footshock of short duration (0.6 mA 0.5 s). The aversiveness of the footshock was apparent via the considerable increase in reactivity during shocking as measured using the shock sensitization paradigm. Moreover, fear conditioning was highly effective in the fear-potentiated startle paradigm, as all groups exhibited significant fear to CS presentations thus indicating effective US-CS associations. Therefore, it is highly likely that the level of shock used in this thesis produced physical and emotive conditions akin to pain, spinal motor reflexes and fear.

Of particular interest is the pain produced by footshock which appears to be processed in the central amygdala. Neugebauer and Li (2002), reported that the central amygdala is receptive to pain that is modifiable by glutamate (Manning, Martin, & Meng,

2003; Neugebauer, Li, Bird, Bhave, & Gereau, 2003; Neugebauer, Li, Bird, & Han, 2004).

Li and Neugebauer (2004) investigated neuronal firing patterns and used these to study pain. They showed that neuronal firing under non-noxious physical conditions such as low-intensity stimulation of skin (via touching with an artist brush or skin pressure that did not result in a withdrawal reflex) was not blocked by the application of AP-5 in the central amygdala. They reported that AP-5 did not affect neural activity to painless stimuli, however, during noxious stimulation (such as a painful skin pinch that produced withdrawal), AP-5 significantly reduced central amygdala neural activity. Thus AP-5 reduced neural firing during noxious stimulation but not during innocuous stimulation (Li & Neugebauer, 2004). These findings could explain the results reported here.

Perhaps consistent with this is pain perceived during footshock, which was blocked by AP-5 thus attenuating the shock sensitization effect. Conversely, when testing for fear during the fear-potentiated startle paradigm, rats were not exposed to painful stimuli, and consequently AP-5 failed to affect fear-potentiated startle. Li and Neugebauer (2004) suggest that NMDA receptors aid pain-related processes. Since pain is absent during conditioned fear expression but not during shock augmented startle, it is possible that AP-5 only affects pain perception.

Experiment 23: The role of AMPA antagonism in fear expression during the fear potentiated startle paradigm.

CNQX abolished the central fear state as shown during the shock sensitization paradigm, and the infusion of NBQX (a AMPA/kainate antagonist similar to CNQX) can prevent fear acquisition (Walker & Davis, 2002). Both these findings suggest a role for

glutamate receptors in fear processing. To further investigate the role of the non-NMDAr antagonist in fear processing CNQX, was infused during the fear potentiated startle paradigm.

Unlike AP-5 which did not affect fear expression, it was expected that CNQX would prevent fear expression. Li and Neugebauer (2004) reported that non-NMDA neurotransmission was involved in processing both noxious and non-noxious stimuli.

To investigate the role of CNQX in fear expression, 10 rats were surgically prepared and after histological verification 8 rats were known to have had cannulae in the central amygdala. Figure 7.2 shows the mean startle amplitudes (±S.E.M) of the saline group from Experiment 22 and this was compared to the CNQX group during the 4 manipulations. The main finding was that CNQX blocked fear potentiated startle during the main drugged fear test.

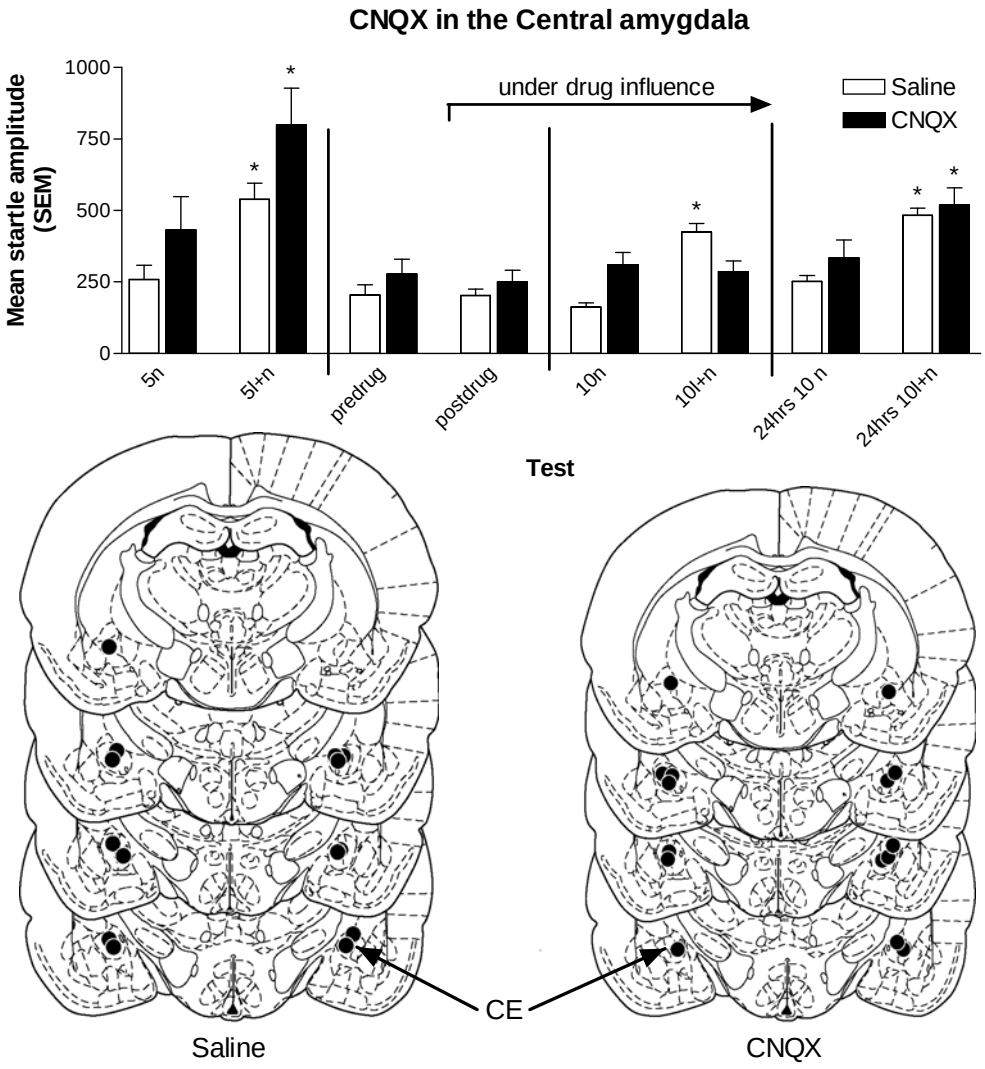

Figure 7.2: Effect of CNQX in fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 4µg/µl CNQX (N=8) after the pre-drug test. This was followed by a fear expression test under drug influence (10n 10l+n) and 24 h later with another fear test (24h 10n-24h 10l+n). Rats infused with CNQX were unable to display fear expression during the 10 noise 10 l+n test. * denotes significant startle increases after CS presentation for each manipulation for each drug, $P<0.05$. Cannulae placements in the amygdala are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

Figure 7.2 illustrates that the rats under the influence of CNQX failed to express fear during the main fear expression test. No significant differences were found between acoustic startle responses before and after CS presentation. A 2 (drug) x 2 (test) repeated measures ANOVA revealed a significant interaction ($F_{1,13}$=27.44, P<0.0001) and a significant main test effect ($F_{1,13}$=18.61, P<0.0008). Simple effects analyses showed that rats in the saline group experienced significant fear (F=42.78, P<0.00001), while the group under influence of CNQX did not (F=0.45, n.s.). These results replicated the findings of Walker and Davis (1997b) who infused NBQX (a potent AMPA/kainate antagonist similar to CNQX) prior to fear expression and found that NBQX significantly reduced the startle response after CS presentation.

Twenty four hours later, both groups showed significant increases between acoustic startle responses before and after CS presentation. A 2 (drug) x 2 (test) repeated measures ANOVA showed a significant main test effect ($F_{1,13}$=20.74, P<0.0005). This test showed that the CNQX effect seen during the main fear test was of a temporary nature. Moreover, both groups still exhibited significant fear to CS presentation indicating that fear must have been present during the main drug test but this was affected by CNQX infusion.

It is unlikely that the CNQX effect was due to a suppression of the startle reflex. Figure 2 depicts no significant differences between pre-test/post-test acoustic startle responses either in the saline or the CNQX group. A 2 (drug) x 2 (test) repeated measures ANOVA failed to indicate a significant effect (F=0.17 n.s.).

Lastly, it is unlikely that during the main fear test the rats did not experience fear prior to drug infusion because both groups displayed significant fear, as was

demonstrated by the short fear test. A 2 (drug) x 2 (test) repeated measures ANOVA revealed a significant main effect for test ($F_{1,13}$=14.33 P<0.002).

Since NMDA and AMPA receptors are involved in pain perception (Li & Neugebauer, 2004) and both AP-5 and CNQX block the shock sensitization effect, it is intriguing to see that AP-5 had no effect on fear expression whereas CNQX did. In fact, CNQX significantly blocked fear expression. Moreover, NBQX also blocks fear acquisition (Walker & Davis, 2002). A possible reason for this was suggested by Li and Neugebauer (2004), who discovered that non-NMDA receptor activation was involved during nociceptive, non-nociceptive and background activity. And this activity could be blocked by NBQX (a potent AMPA/kainate antagonist, similar to CNQX). Thus, the blocking of the AMPA receptor reduced both nociceptive and non-nociceptive neurotransmission. Therefore, it becomes difficult to determine the precise role of AMPA neurotransmission in the shock sensitization paradigm, fear acquisition and fear potentiated startle. But we can conclude that during the fear potentiated startle paradigm it is unlikely that CNQX suppresses pain. Since fear expression to a CS does not involve noxious stimulation, CNQX may still affect fear expression through attenuation of responses to non-painful stimuli. Thus CNQX may affect the processing of the CS via inhibition of non-NMDA neurotransmission. To clarify the role of AMPA receptors in fear expression and shock sensitization a dose response study would show the level of AMPA receptor involvement.

Experiment 24: The role of protein synthesis inhibition in fear expression during the fear potentiated startle paradigm.

The central amygdala is a site for plasticity, involving long-term potentiation and protein synthesis (Bahar, Samuel, Hazvi, & Dudai, 2003; Wilensky, Schafe, Kristensen, & LeDoux, 2006). Long-term potentiation includes the formation of new synapses thus making neurotransmission along particular pathways more efficient. These structural changes involve protein synthesis which can be blocked by the novo protein synthesis inhibitor anisomycin. Some evidence exists for a role for long term potentiation in the central amygdala (Bahar, Samuel, Hazvi, & Dudai, 2003; Goosens & Maren, 2003). The infusion of anisomycin prior to conditioned taste aversion prevents its acquisition (Bahar, Samuel, Hazvi, & Dudai, 2003). However, the infusion of anisomycin into the central amygdala during the shock sensitization paradigm had no effect on the central fear state produced by footshock. This was similar to the results found in the basolateral amygdala. But the basolateral amygdala was affected during the fear potentiated startle paradigm. A significant inhibitory effect was found, during the '24 hours later' test when anisomycin was infused into the basolateral amygdala (See Experiment 14) and rats failed to express fear 24 hours post anisomycin infusion. It was postulated that this was due to activation of the CS memory during the main fear test with anisomycin by perhaps introducing new structural changes that were measurable the next day. Thus the question asked was, can the infusion of anisomycin into the central amygdala affect fear expression? Ten rats were surgically prepared and after histological verification 3 rats were excluded from analyses.

The infusion of the novo protein synthesis inhibitor anisomycin into the central amygdala produced results difficult to interpret during the main drugged fear testing phase. Figure 7.3 illustrates the mean startle amplitudes from the control group and these were compared to the anisomycin group during the 4 manipulations. Anisomycin-infused rats showed no significant effect during the main fear test, but was this a drug effect?

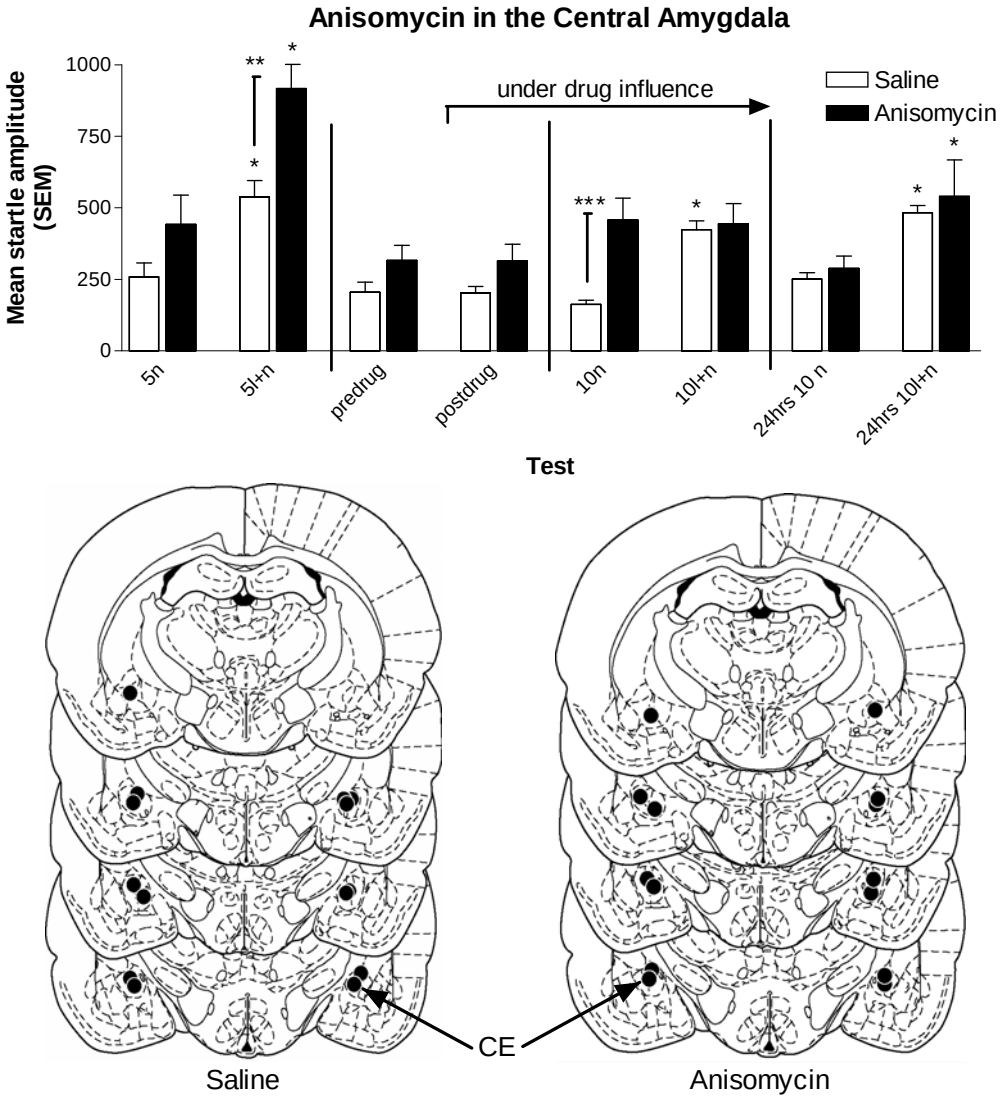

Figure 7.3: Effect of anisomycin in fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a predrug /postdrug (20n, 20n) test where rats were infused with saline (N=7) or 80µg Anisomycin (N=7) after the predrug test. This was followed by a fear expression test under drug influence (10n 10l+n), and 24 h later with another fear test (24h 10n-24h 10l+n). Rats infused with anisomycin were affected, but see discussion. * denotes significant increases in startle amplitudes between n and l+n within each drug, $P<0.05$. ** denotes a significant difference between saline and anisomycin groups during 5l+n trials, anisomycin group more reactive, $P<0.05$. *** denotes a significant difference between saline and anisomycin groups during the 10 n trials under drug influence, anisomycin group more reactive, $P<0.05$. Cannulae placements in the central amygdala are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

In Figure 7.3 it is evident that the rats in the anisomycin group were more active during the first two tests (short fear test and pre-drug/post-drug) than the saline group. In particular, a significant difference was exhibited during the 10 noise trials in the main fear test. However, the rats infused with anisomycin failed to express significant fear-potentiated startle during the drugged fear test. A 2 (drug) x 2 (test) repeated measures ANOVA revealed a significant interaction ($F_{1,12}$=37.31 P<0.00005) and a main effect for test condition ($F_{1,12}$=30.64 P<0.0001). Simple effects analyses showed a strong effect for saline (F=67.79, P<0.000003), but no effect for anisomycin (F=0.16, n.s.).

Because of the heightened reactivity of the anisomycin infused rats during the 10 noise bursts, it was difficult to draw a sound conclusion about the effect of anisomycin on fear-potentiated startle. One possible reason was that a ceiling effect prevented the rats from responding with a further increase in startle to the CS presentation, since both the saline-and anisomycin-infused rats showed similar reactivity to the CS and thus no effect of anisomycin on fear expression. Conversely, since the anisomycin group appeared more reactive during the previous tests it could be argued that anisomycin attenuated fear responses to CS presentation, because no significant differences in acoustic startle amplitudes were found before or after CS presentation. If the enhanced startle noticed during the 10 noise bursts was due to a drug effect, than this effect was not exacerbated after CS presentation. This suggests an inhibitory role of anisomycin on fear expression, but this experiment would need to be repeated for a more conclusive result.

Twenty four hours later the anisomycin group exhibited behaviour similar to the saline group. Here both groups showed similar magnitudes in startle amplitude before and after CS presentation. A 2 (drug) x 2 (test) repeated measures ANOVA showed a

significant main test effect ($F_{1,12}$=21.64, P<0.0005). Simple effects analyses showed both saline and anisomycin groups exhibited fear-potentiated startle (F=9.95, P<0.008, F=11.72, P<0.005, respectively).

The augmented startle seen during the 10 noise bursts in the main fear test was most likely not a result of anisomycin infusion, since no significant differences were found between pre/drug and post/drug infusion. No significant effects were found using a 2 (drug) x 2 (test) repeated measures ANOVA ($F_{1,12}$=0.00, P<0.99). However, a nearly significant effect for drug occured ($F_{1,12}$=4.68, P<0.051) and, as can be seen in Figure 7.3, rats in the anisomycin group overall were more responsive to noise.

The lack of fear-potentiated startle demonstrated during the main fear test in the anisomycin group was not due to a fear acquisition failure, as significant increases in startle were recorded during the test 24 hour later, and also during the short fear test, since both groups displayed significant fear-potentiated startle during the short fear test. As can be seen in Figure 7.3, rats in the anisomycin group overall were more reactive. A 2 (drug) x 2 (test) repeated measures ANOVA showed a significant main test effect ($F_{1,12}$=27.04 P<0.0002) but also a significant main drug effect ($F_{1,12}$=6.75 P<0.02). Lastly, it was unlikely that the lack of results was due to the concentration of anisomycin or the paradigm, because 80 µg/µl was effective in preventing fear expression during the 24 hour later test in the basolateral amygdala (see Section 3 Experiment 14).

Cautious interpretation of the results suggests that anisomycin may not affect fear expression when infused into the central amygdala. However, anisomycin prevents protein synthesis necessary for structural changes within postsynaptic neurons (Okulski, Hess, & Kaczmarek, 2002). In addition, anisomycin may affect the release of

neurotransmitters such as norepinephrine and acetylcholine (Gold, 2003; McGaugh, 2004). Acetylcholine receptors are found in abundance in the central amygdala and are located in conjunction with GABAergic receptors and are involved in fear expression (van der Zee, Roozendaal, Bohus, Koolhaas, & Luiten, 1997). Furthermore, Canel and Gold (2007) suggested that injections of anisomycin can have an immediate effect on memory recall when infused into the amygdala. Consequently, these reasons suggest that anisomycin may affect fear expression but, because the above-presented results did not clearly show this, some caution is required in concluding that this is indeed the case. This experiment would benefit from being repeated to improve the clarity of the results.

Experiment 25: The role of GABA neurotransmission during fear potentiated startle.

The next drug infused into the central amygdala was the GABA agonist muscimol. Functional inactivation of the central amygdala by infusion of muscimol prevented the expression of freezing behaviour (Holahan & White, 2004; Wilensky, Schafe, Kristensen, & LeDoux, 2006), suggesting a role for the central amygdala in fear expression. In Section 4 it was clear that muscimol, infused at a low concentration, failed to suppress the shock enhanced acoustic startle effect. The failure of muscimol to suppress the central fear state does not necessarily predict a failure in fear expression. Fear expression also entails a cognitive function such as attention, memory and activation of the startle circuit. It was expected that if a cognitive component was affected by muscimol than we would see a lack of fear expression. To determine if muscimol could suppress fear expression, 10 rats were surgically prepared with cannulae aiming for the central amygdala. One rat failed histological verification and was not used for analyses. Figure 7.4 illustrates the

results of the infusion of 0.005µg/µl muscimol and saline on conditioned fear expression as shown by as startle amplitude. Cannulae placements are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

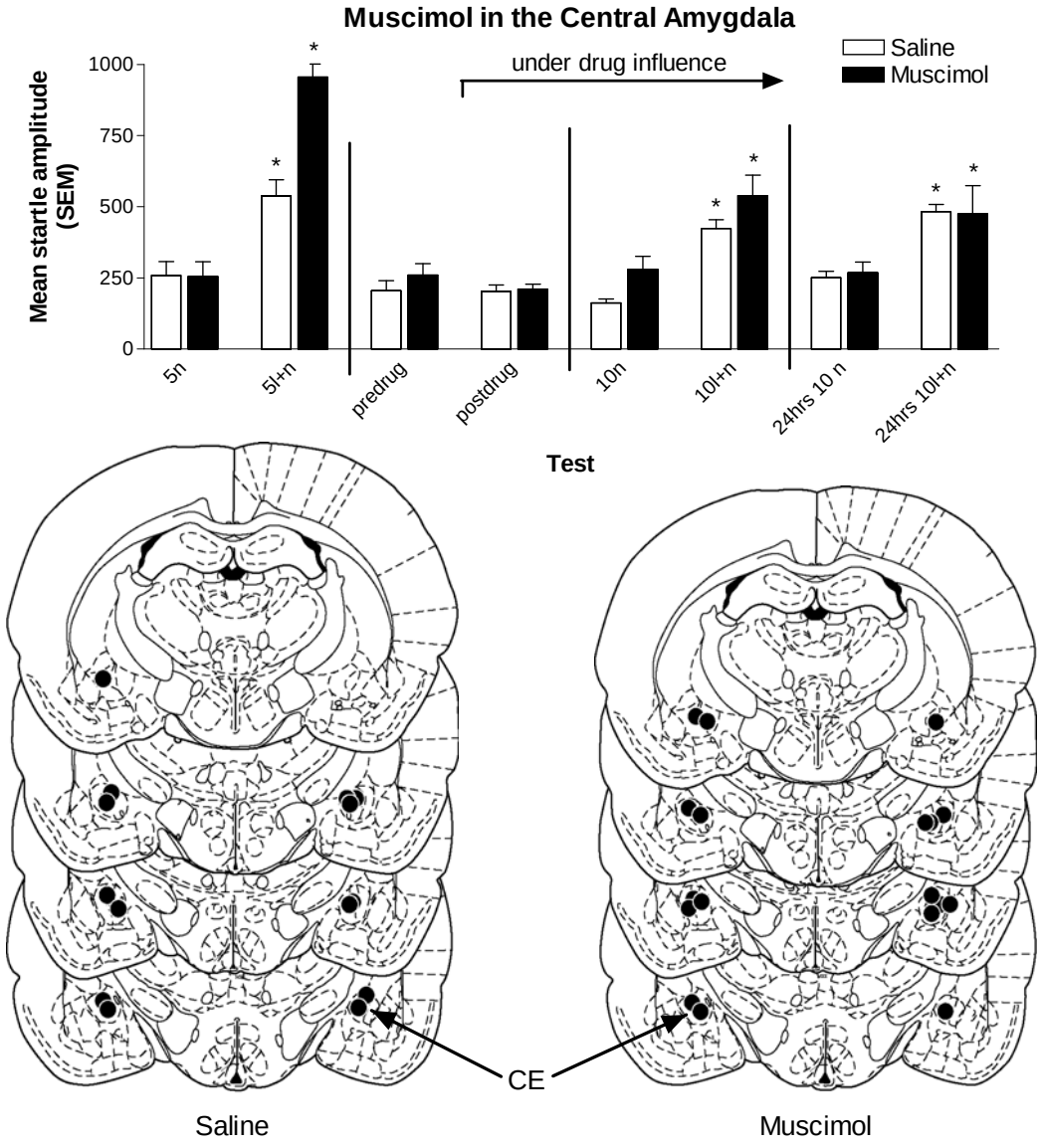

Figure 7.4: Effect of muscimol on fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a predrug /postdrug (20n, 20n) test where rats were infused with saline (N=7) or 0.005µg/µl muscimol (N=9) after the predrug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n, 24h 10l+n). Rats infused with muscimol were unaffected in their ability to express fear during the 10 noise 10 l+n test. * denotes significant increases in acoustic startle responses before and after CS presentation, within each drug, $P<0.05$. Cannulae placements in the central amygdala are depicted on schematics adapted from Paxinos and Watson (1998) rat brain atlas. CE is central amygdala.

The main finding illustrated in Figure 7.4 was that muscimol had no effect on fear expression when infused into the central amygdala. Both the saline and the muscimol group showed a significant increase in startle amplitude after CS presentation. A 2 (drug) x 2 (test) ANOVA showed a significant main test effect ($F_{1,14} = 47.39$, p=0.000008). Simple effects analyses showed that infusion of saline (F= 21.22, p=0.0004) or muscimol (F=26.87, p=0.0001) did not suppress fear expression.

Both groups were tested 24 h later and exhibited significant fear. A 2 (drug) x 2 (test) ANOVA showed a main test effect ($F_{1,14} = 23.77$, p=0.0002). These results suggest that the manipulation of drug infusion and the fear test under the drug's influence did not affect fear expression the following day. Similarly, the infusion of saline or muscimol prior to the main fear test did not interfere with the acoustic startle response. A 2 (drug) x 2 (test) repeated measures ANOVA showed a non-significant difference (F=0.74 between the pre-drug/post-drug noise bursts. Finally, the short fear test showed that both groups experienced fear prior to the drug testing procedure. A 2 (drug) x 2 (test) repeated measures ANOVA showed an interaction ($F_{1,14} = 12.67$, p=0.003), and two main effects, drug ($F_{1,14}$=5.57, p=0.033) and test ($F_{1,14}$=68.48, p=0.000001). The main drug effect could be attributed to the greater reactivity of the muscimol group during the short fear test.

This experiment showed that a small dose of muscimol (0.005 µg/µl) failed to suppress fear expression in the central amygdala. Arguably the dose level was too low but it was nevertheless sufficient to control fear in the basolateral amygdala during both shock sensitization and fear expression. This suggests that $GABA_a$ receptors in the central amygdala (Pedersen, Scheel-Kruger, & Blackburn-Munro, 2007) were not

involved in fear expression, as measured by fear potentiated startle. The use of a low dose showed that the results reported by earlier investigators by (Holahan & White, 2004; Wilensky, Schafe, Kristensen, & LeDoux, 2006) did indeed involve functional inactivation and that this may have affected the overall performance of the central amygdala. Section 4, Experiment 19 showed that activation of the central fear state was not affected by a low dose of muscimol; the above results showed that muscimol does not appear to affect cognitive functioning either.

Experiment 26: The role of the dopamine D1 agonist in fear expression during the fear-potentiated startle paradigm.

Dopamine neurotransmission has been implicated in the acquisition and expression of conditioned place preference. For example, the infusion of 2µg/µl SKF 38393 into the central amygdala blocked the expression of morphine induced place preference (Zarrindast, Rezayof, Sahraei, Haeri-Rohani, & Rassouli, 2003). The central amygdala contains dopamine neurons and these are activated during emotional and aversive events (Fudge & Emiliano, 2003). However little is known about the effects of the D1 agonist SKF 38393 on the central amygdala during fear expression. As was shown in Section 4, Experiment 21, infusion of the D1 agonist SKF38393 into the central amygdala failed to reduce footshock associated fear-arousal. This failure to reduce the central fear state may not necessarily predict a failure in fear expression during the fear potentiated startle paradigm because dopamine may affect fear expression via mnemonic factors. To investigate the effects of SKF 38393 on fear expression, 10 rats were surgically prepared with cannulae aiming for the central amygdala. After histological verification, 1 rat was excluded from the analyses. Figure 7.5 illustrates the effect of infusion of saline and SKF

38393 during fear expression as expressed by the mean startle amplitude. Cannulae placements in the central amygdala of the saline and SKF 38393 group are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

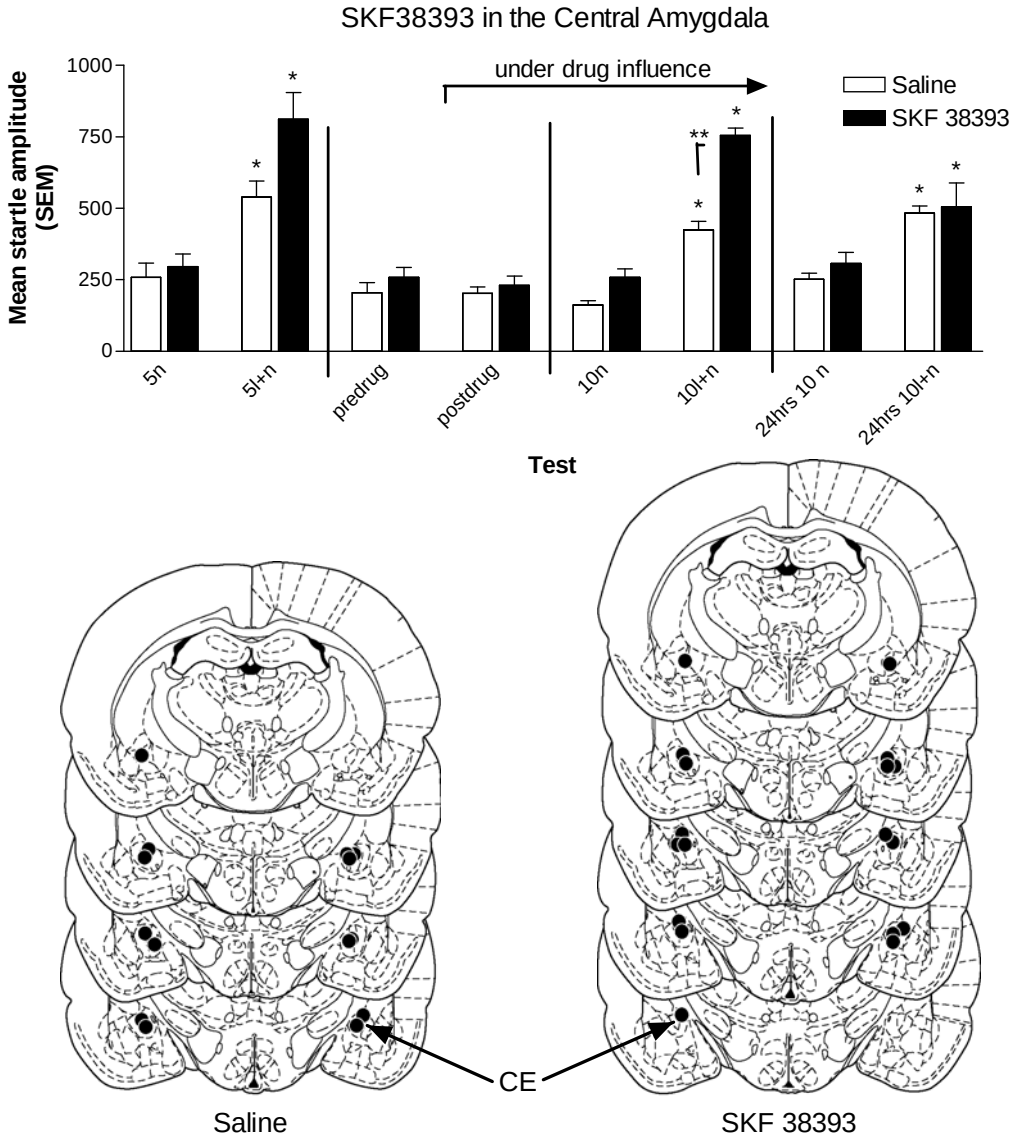

Figure 7.5: Effect of SKF 38393 in fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 4µg/µl SKF38393 (N=9) after the pre-drug test. Followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with another fear test (24h 10n 24h 10l+n). * denotes significant increases in startle amplitudes after CS presentation within each drug group, $P<0.05$. ** denotes rats infused with SKF38393 displayed enhanced fear expression as compared to saline during the 10 noise 10 l+n test, $P<0.05$. Cannulae placements in the central amygdala of the saline and SKF 38393 group are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

Figure 7.5 demonstrates that rats under the influence of SKF38393 during the main fear expression test, displayed higher startle amplitudes as compared to saline-treated rats. A 2 (drug) x 2 (test) repeated measures ANOVA showed a significant interaction ($F_{1,14}$=18.65, p<0.0007) and two highly significant main effects, namely a drug effect ($F_{1,14}$=60.56, P<0.000002), and a test effect ($F_{1,14}$=194.12, P<0.000000). Simple effects analyses showed that for both the saline and SKF 38393 groups there were significant increases in startle responses during the 10L+N condition, compared to the 10 noise (F=41.07, P<0.00001, F=190.37, P<0.0000 respectively), thereby indicating a significant fear effect. However, the SKF 38393 group had an even greater increase in startle amplitude as compared to the saline group. Simple effects analyses between saline and SKF 38393 startle scores for the 10L+N data showed a significant increase (F=66.29, P< 0.000001). This increase in startle was interpreted as an index of fear thus suggesting that SKF 38393 enhanced the expression of fear more than for the saline group. These results were comparable to the infusion of the D1 agonist SKF 82958 which increased freezing behaviour to CS presentations compared to saline infusions (Guarraci, Frohardt, & Kapp, 1999).

The present results extend those reported by (Borowski & Kokkinidis, 1998) who observed that intraperitoneal injections of cocaine, amphetamine (both dopamine agonists), and SKF 38393 prevented fear extinction. This extinction deficit was thought to be due to the drugs' ability to activate the CS-US connections. The non-reinforced CS presentations continued to elicit substantial fear, thus preventing extinction. The presentation of a CS not only elicits fear but also attentional processes (Gallagher & Holland, 1994), and dopaminergic agonism can potentiate the motivational effects of the

CS (Wise, 2004). Thus the rats may have directed their attention more exclusively to the CS and so may have generated a greater cental fear state. Furthermore, the presentation of a CS that predicts pain will increase attention as compared to a CS predicting safety (Van Damme et al., 2004). In humans, cognitive tasks release dopamine in the amygdaloid complex (Fried et al., 2001). In effect this means that dopamine D1 receptors are more likely to be involved in attention to the CS then in perception of aversive stimuli (El-Ghundi, O'Dowd, & George, 2001; Gallagher & Holland, 1994; Granon et al., 2000). Indeed, the shock sensitization paradigm lacks specific cues that could draw attention to the presentation of the non-signalled shock. No difference in acoustic startle responses after footshock presentation between the control and the SKF 38393 group was observed. In contrast, during the fear-potentiated startle paradigm, CS presentation does predict the onset of an aversive stimulus, and significant enhanced fear-potentiated startle was seen after CS presentation.

The increase in startle magnitude seen during the main fear test returned to an equivalent level as the control group 24 h later. A 2 (drug) x 2 (test) ANOVA with the repeated measures on the 10n, 10l+n test showed a significant main test effect ($F_{1,14}$=29.22, P<0.00009). Simple effects analyses revealed that rats in the saline and SKF 38393 groups both showed significant increases in startle responses (F=15.10, P<0.001, F=14.19, P<0.002, respectively). This test revealed that the increase in behavioural responses shown during the drugged test was not carried over to the next day.

If the drug-induced startle-enhancing effects noticed during the main fear-potentiated startle test was due to an increase in locomotion, this was not supported by

startle data collected during the pre-drug/post-drug test. The actual infusion of saline or SKF 38393 did not modify the behavioural responses to acoustic stimuli, as a 2 (drug) x 2 (test) repeated measures ANOVA showed no significant effects (p=0.6). Thus, the extreme startle response during the fear test could not be attributed to an increase in general locomotion. Zarrindast et al (2003) also noted that 2 and 4 μg SKF 38393 infused into the central amygdala did not affect locomotor activity during testing for place preference. Supporting the proposition that SFK 38393 may enhance attention to CS.

It could be argued that the enhanced startle effect seen during the drugged fear test was a pre-existing condition, because rats in the SKF 38393 group showed a greater magnitude in startle response during the short fear test. However, both groups exhibited significant increases in the startle response during the short fear test. A 2 (drug) x 2 (test) ANOVA revealed a significant interaction effect ($F_{1,14}$=5.49, P<0.03) and main test effect ($F_{1,14}$=62.76,P<0.000002), but, no significant main drug effect ($F_{1,14}$=3.58, p=0.08 n.s.). This indicated that although the SKF 38393 group was somewhat more responsive to the startle stimulus, this was not significantly different from the saline group. Thus it is unlikely that the enhanced startle effect seen during the drugged fear test was entirely due to overall enhanced responding. Consequently the results suggest that agonism of the dopamine D1 receptor in the central amygdala increased fear-potentiated startle.

Experiment 27: The role of the D2 agonist quinpirole in fear expression during the fear potentiated startle paradigm.

The central amygdala also contains D2 receptors but their role in fear expression as measured by startle is unknown. Nevertheless, D2 antagonism attenuated fear acquisition but not fear expression as measured by freezing behaviour (Guarraci, Frohardt, Falls, &

Kapp, 2000). Thus blocking of the D2 receptor had little effect on the expression of freezing behaviour. Conversely, the effect of stimulating the receptor has not been investigated. The results from the shock sensitization paradigm showed that quinpirole had no effect on immediate fear arousal produced by foot-shock. This suggests that the central fear state produced by footshock was not affected by quinpirole. However, the possibility that quinpirole may affect a more mnemonic process could not be ruled out. Since the D1 agonist SKF 38393 enhanced startle responses during the main fear test in the previous experiment it was postulated that quinpirole may also affect fear expression as measured by an increase in startle. Often D1 and D2 receptors work together on the same neural level (Meloni & Davis, 1999; Missale, Nash, Robinson, Jaber, & Caron, 1998). To investigate the role of quinpirole on fear expression, 10 rats were surgically prepared with cannulae aiming for the central amygdala. Two of these rats failed histological verification and their data were not included in the analyses. Figure 7.6 illustrates the results of saline and quinpirole infusions into the central amygdala. The startle data is represented as the mean startle amplitudes measured during the four manipulations. Cannulae placements in the central amygdala of the saline and quinpirole groups are depicted on schematics adapted from Paxinos and Watson's (1998) rat brain atlas.

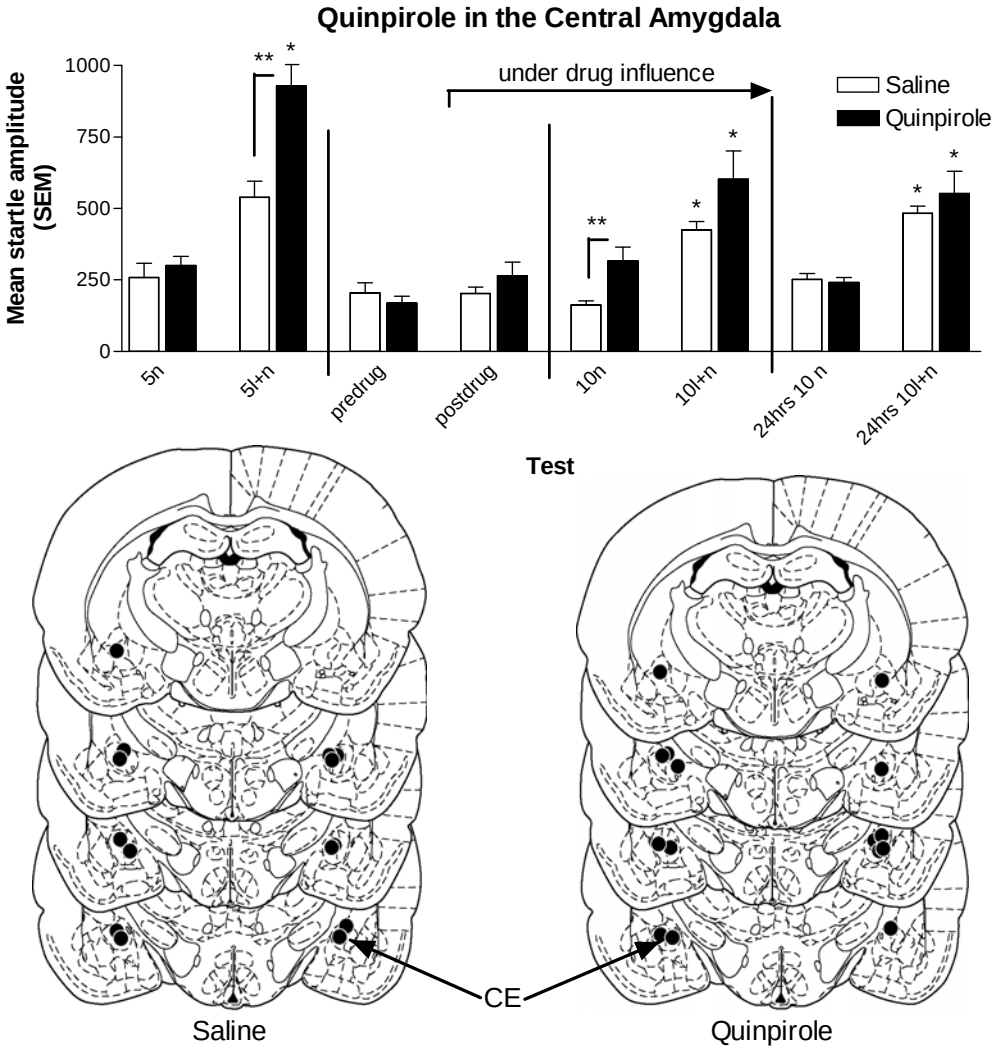

Figure 7.6: Effect of quinpirole in fear potentiated startle expression in the central amygdala.

Mean (±S.E.M.) acoustic startle amplitudes measured during a short fear test (5n, 5l+n), a pre-drug /post-drug (20n, 20n) test where rats were infused with saline (N=7) or 3µg/µl quinpirole (N=8) after the pre-drug test. This was followed by the main fear expression test under drug influence (10n 10l+n), and 24 h later with a final fear test (24h 10n-24h 10l+n). Rats infused with quinpirole were unaffected and displayed increased startle responding during the 10 n, 10 l+n test. * denotes significant increases in startle amplitudes after CS presentation within each drug group, $P<0.05$. ** denotes a significant difference between saline and quinpirole during the 5 L+N trials and during the 10 Noise trials, $P<0.05$. Cannulae placements in the central amygdala are depicted on schematics adapted from Paxinos and Watsons (1998) rat brain atlas.

As outlined in Figure 7.6, the infusion of quinpirole failed to affect startle reactivity during the main fear test. A significant difference between acoustic startle responses before and after CS presentation was found. A 2 (drug) x 2 (test) repeated measures ANOVA with the repetition on the 10n, 10l+n test showed a significant main effect for test condition ($F_{1,13}$=44.46, P<0.00001), and for drug ($F_{1,13}$=4.78, P<0.047). Simple effects analyses showed that the drug effect was attributable to the difference between the noise groups (F=8.18, P<0.01), but not between the light + noise groups (F=2.65, n.s.). Thus, a significant difference was found between saline and quinpirole groups during the 10 noise trials, but this difference did not occur during the CS+ noise trials. Both the saline and quinpirole groups showed a similar increase in the startle response, suggesting that quinpirole had no effect on fear expression as measured by startle.

Twenty four hours later, both groups still showed significant increases in startle after the CS presentation. A 2 (drug) x 2 (test) repeated measures ANOVA with the repetition on the 10n, 10l+n test showed a large main test effect ($F_{1,13}$=16.70, P<0.001). Simple effects analyses showed that both saline and quinpirole groups displayed a significant increase in startle after light presentation (F=14.36, P<0.002, F=29.56, P<0.0001, respectively). This test revealed that quinpirole had no discernable effect 24 hours after infusion.

Intraperitoneal injections of quinpirole have been shown to enhance stereotyped locomotion (Eilam, Clements, & Szechtman, 1991) but a similar effect was not seen during the pre-drug/post-drug test. No significant effect was found according to a 2 (drug) x 2 (test) repeated measures ANOVA with the repetition on the test variable ($F_{1,13}$=2.41, P=0.14, n.s.). This suggested that infusion of quinpirole into the central

amygdala does not affect locomotion (as seen in the study of Eilam, Clements, & Szechtman, 1991).

Figure 7.6 shows that during the short fear test (5n, 5 l+n), rats in the quinpirole group were significantly more reactive than the saline group when presented with the CS. Analyzing the short fear test with a 2 (drug) x 2 (test) repeated measures ANOVA with the repetition on the test variable showed a significant test effect ($F_{1,13}$=16.70, P<0.001) and a near significant drug effect ($F_{1,13}$=4.24, P=0.06). Simple effects analyses showed that the quinpirole group exhibited significant fear-potentiated startle (p=0.0012), but the saline group did not (p=0.107). However, this was the same saline group that had been used in the above comparisons where fear was found to be statistically significant. The lack of statistical significance was due to the large fear effect in the quinpirole group. A subsequent t-test showed that the startle amplitude to the CS+ noise was significantly higher compared to the noise alone variable (t_{12} =-3.74, P<0.01, one tailed).

The lack of effect in the main fear test was not due to the concentration of quinpirole as it successfully blocked fear expression in the basolateral amygdala under the same experimental conditions. Thus, quinpirole did not affect the central fear state or any measurable cognitive functioning, as measured by fear-potentiated startle. The failure of quinpirole to affect fear expression as measured by a change in the startle response showed that D2 receptor agonism had a limited role in the central amygdala for fear expression.

Summary of results

Experiment 22 demonstrated that bilateral infusion of AP-5 into the central amygdala did not affect fear expression. This effect was interpreted as a ‘pain’ effect.

That is, by a process of elimination, AP-5 reduced immediate fear arousal in the central amygdala which was suggested to be a reduction in pain perception. But fear expression to a CS does not involve pain and thus AP-5 did not affect fear expression.

Experiment 23 showed that blocking AMPA receptors prevented fear expression when CNQX was bilaterally infused into the central amygdala. This was interpreted as a general blockade of AMPA receptors present in the central amygdala.

Experiment 24 suggested that anisomycin may not have affected the fear expression test.

In Experiment 25, bilateral infusion of muscimol demonstrated that GABA was not significantly involved in fear expression in the central amygdala.

Experiment 26 showed that D1 agonism significantly enhanced fear expression to CS presentation. This was interpreted as a cognitive effect.

Experiment 27 showed that D2 agonism did not affect fear expression and this was interpreted as D2 receptors not being involved in fear processing.

GENERAL DISCUSSION.

In this thesis five questions were asked. The first one investigated the effects of footshock on startle expression. The behavioural studies showed that footshock could significantly amplify acoustic startle responses; this increase was interpreted as an index of a central fear state. The second question was: which neurotransmitters were involved in suppressing this central fear state in the basolateral amygdala? It was found that GABAergic neurotransmission was involved. The third question was: is this central fear state the same as the fear state seen during CS presentation in a fear-potentiated startle paradigm? The answer appears to be yes. GABAergic neurotransmission was the only system investigated that suppressed the central fear state during both paradigms. It was thus concluded that it is the central fear state produced by footshock that was affected by a GABAergic system, and not pain or spinal reflexes.

The fourth question was: is the central amygdala involved in the expression of the central fear state produced by footshock? Yes it is. It appears to be controlled by a glutamatergic system, since both NMDA and AMPA antagonists prevented fear expression. This was interpreted as a deficit in pain processing but not fear. The final question was: do the same neural systems control shock elicited and CS elicited fear in the central amygdala. Yes and no. NMDA antagonism could prevent fear expression to footshock but not to CS presentation; conversely AMPA antagonism could prevent fear expression to both stimuli. These results were interpreted as a NMDA system controlling

pain produced by footshock, and AMPA having a dual role, both preventing pain and normal neural transmission.

The behavioural section showed in Experiment 1 that footshock reliably increased startle responses. This increase was interpreted as an expression of an internal fear state. Experiments 2-5 showed that shock sensitization was not influenced by external prompts such as context or explicit cues or by duration of chamber exposure before foot-shock presentation. Evidence for the failure of contextual conditioning was found in Experiment 2. Rats that had been exposed to the chamber and to acoustic stimuli prior to 10 foot-shocks showed no increase in startle during testing the next day. This suggests that foot-shock did not elicit contextual conditioning as measured by startle. Moreover, as shown in Experiment 3, by exposing the rats to an explicit cue, fear-conditioning still failed. By abolishing chamber pre-exposure in the immediate shock exposure experiment (Experiment 4) rats still showed foot-shock enhanced startle. Thus, whether the rats were shocked immediately or 5 minutes later after placement into the chamber, no immediate startle deficit was evident. This indicated that the startle increase was not due to the formation of associations between context and footshock.

And lastly, over-exposure to the environment can inhibit learning, and in Experiment 5 it was shown that if shock sensitization is a form of contextual conditioning rats should not react to noise after foot-shock exposure. After three days of 20 minute exposures to the chambers, rats still showed augmented startle after footshock. The converging evidence of the five behavioural experiments showed that shock sensitization is a reasonable paradigm to investigate unconditioned immediate fear arousal produced by footshock.

Foot-shock elicits reflex/pain and emotive responses (Borszcz, 1993, 1995; Borszcz & Leaton, 2003; Crown, King, Meagher, & Grau, 2000). Pairing a CS and an US (footshock) allows the CS to acquire some of the US properties, namely the emotive response (Davis, 1986). During CS presentation this response is said to be reactivated and measurable via an increase in startle. It was speculated that the emotive response has a neural representation. This was investigated firstly in the basolateral amygdala and later in the central amygdala. Both these areas play important roles in fear arousal, but via different neural systems (Pare, Quirk, & LeDoux, 2004).

Comparisons between central fear states produced by foot-shock and CS presentation in the basolateral amygdala.

In this thesis, two paradigms were compared to investigate the central fear state. The paradigm representing unconditioned fear was the shock sensitization of acoustic startle. Rats exposed to 20 white noise bursts followed by 10 rapid foot-shocks followed by 20 white noise bursts show a 100% increase in startle after foot-shock. This increase has been shown to be an index of a central fear state (Davis, 1989).

The second paradigm, representing conditioned fear, was a classical fear conditioning paradigm (Davis, 1986). Rats were well trained to acquire the CS-US association. This acquisition was tested at a later date via CS exposure followed by acoustic startle probes. A significant increase between acoustic startle before and after CS presentation was judged to be an index of fear (Davis, 1986).

These two paradigms have in common a central fear state, which is measured via the same response, namely startle. Startle is an ideal measure for fear responses because the startle circuit is mediated via amygdaloid inputs to the caudal pontine reticular

nucleus (Fendt & Fanselow, 1999; Pilz, Carl, & Plappert, 2004). This is a very short circuit mediated by no more then five nuclei (Davis, Gendelman, Tischler, & Gendelman, 1982). More importantly, the magnitude of the startle response is modified by amygdaloid inputs (Davis, Gendelman, Tischler, & Gendelman, 1982; Koch & Schnitzler, 1997). Moreover, the startle reflex is also apparent in humans and is not under intentional control (Grillon & Baas, 2003), making the startle response a useful index for fear expression.

The main differences between immediate fear arousal by foot-shock and fear arousal by a CS were that, during footshock presentations rats displayed motor-reflex activity and potentially experienced pain (Borszcz, 1995). The motor-reflex was measured during footshock exposure in the shock sensitization paradigm. Pain was not directly measured but was inferred from the literature available on rodents and humans (Crown, King, Meagher, & Grau, 2000). Humans report feelings of pain when receiving electric shocks of a similar level to that used on the rats (Tursky, 1973). In rats, shock-enhanced startle can be reduced by the injection of an analgesic without affecting the acoustic startle reflex, thereby indicating pain during shock exposure (Chen, Ho, & Liang, 2000). In addition, measures of vocalization after shock indicate pain enhancement (Crown, King, Meagher, & Grau, 2000).

Neither shock induced motor reflex nor pain were evident during CS presentation. There is evidence that rats do not show spontaneous motor-reflexes during CS presentation (Davis, 1989) but do show freezing behaviour. Moreover, physical pain is not experienced during CS exposure but the fear of pain potentially is (Borszcz, 1995).

Another main difference between the two paradigms was the mnemonic component. The five behavioural studies showed little evidence of mnemonic activity during the shock sensitization paradigm. However, during CS exposure, higher cognitive functions must have been triggered (Davis, 1986). This was to be expected, because during CS presentation, the animals must have recalled the US/CS association for them to be able to express fear to the CS and show augmented startle during testing. Thus, in this manner it is possible to delineate the effect of fear arousal from foot-shock and also from higher cognitive functions. The overlap between the two paradigms is the central fear state.

One way of investigating the central fear state was to suppress it. The suppression could be carried out by pharmacological means. For example, ingestion of benzodiazepines attenuated fear expression (Davis, 1979) but also affected alertness (Bitsios, Philpott, Langley, Bradshaw, & Szabadi, 1999). Thus confounding variables masked the specific effects. A more unambiguous solution is to pharmacologically influence a brain area. The first area investigated was the basolateral amygdala because this is where the CS/US associations are formed and a US representation may be processed (Rogan, Staubli, & LeDoux, 1997b; Romanski, Clugnet, Bordi, & LeDoux, 1993; Sigurdsson, Doyere, Cain, & LeDoux, 2007). The second area was the central amygdala. This area also receives unconditioned stimulus information, particularly that relating to pain information (Li & Neugebauer, 2004; Manning, 1998; Manning, Martin, & Meng, 2003; Neugebauer & Li, 2003; Pare, Quirk, & LeDoux, 2004).

It was hypothesised that the neural system involved in the central fear state produced by footshock was the same as the central fear state produced by CS exposure.

Thus, if the central fear state that was elicited during the shock sensitization paradigm was the same as that produced to CS, then neurotransmitters involved in shock sensitization will also be involved in fear-potentiated startle. But not all neurotransmitters that block fear-potentiated startle will block shock sensitization.

The central question was, are both these fear states controlled by the same neural systems?

To investigate this, neural function was inhibited via the infusion of either a $GABA_a$ agonist or one of two glutamate antagonists. Furthermore, dopamine agonism and protein synthesis were explored.

The function of the GABAergic system in the basolateral amygdala.

The first major finding was that a GABAergic system in the basolateral amygdala was involved in immediate fear arousal produced by foot-shock (van Nobelen and Kokkinidis 2006). The inhibitory function of GABA significantly affected fear responses. This was shown by the dose response curve illustrating that muscimol concentrations as low as 0.001μg/μl reliably suppressed the central fear state.

According to Borszcz (1993, 1995), shock elicits three components, namely physical (reflex and pain) and an emotive element. The results showed that muscimol did not affect the physical properties produced by footshock. That is, the startle reflex was not affected by muscimol. Pain was more difficult to exclude but it was unlikely to have been involved because the basolateral amygdala does not appear to receive pain signals, unlike the central amygdala (Manning, 1998). Thus it was concluded that muscimol affected the emotive element.

If the above stated conclusion is correct, then muscimol should also block the expression of fear-potentiated startle, and it does. A small dose of 0.005µg/µl muscimol affected the central fear state when infused prior to CS presentation. Rats failed to express fear. This failure was not due to ataxic effects of muscimol on startle behaviour. Throughout the paradigm, rats showed significant responses to acoustic startle probes. The startle responses were augmented by CS presentation in the short fear test to demonstrate that rats experienced fear prior to drug infusion. In addition, rats also showed clear fear expression to CS presentation twenty four hours after drug infusion. This indicated that the inhibitory muscimol effect was of a temporary nature.

As suggested earlier, the overlap between the central fear state produced by footshock and the central fear state produced by CS presentation may be controlled by the same neural system. One interpretation of the data was that the GABAergic system prevented the expression of fear in both. Thus, a small dose of muscimol could effectively prevent fear expression after footshock and CS presentation via a reduction in a central fear state (Chi 1965). The fear expression medium, startle, was not affected in either paradigm. However, during the fear-potentiated startle paradigm it was not easy to exclude the possibility that higher cognitive functions were unaffected by the small muscimol dose. For example, attention to and memory of the CS may have been attenuated and thus induced the fear expression deficit. However, these potentially confounding factors were difficult to empirically eliminate.

It is likely that GABA is not specifically involved in memory and attention for the following reasons. Once fear acquisition has been established via a long-term potentiation process it is the maintenance of this process that represents a form of memory (Izquierdo,

1994). It is accepted that for a drug to be unequivocally involved in developing fear associations via long-term potentiation it should prevent fear acquisition when infused prior or directly after US/CS presentations. Moreover, this drug should not prevent fear expression when infused prior to fear recall (Kim & Jung, 2006). Muscimol infusions do interfere with the development of fear associations when infused prior to US/CS exposure (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997; van Nobelen & Kokkinidis, 2006) but do not interfere with consolidation (Wilensky, Schafe, & LeDoux, 1999, 2000). And muscimol interferes with fear expression when infused prior to fear recall (Helmstetter & Bellgowan, 1994) and see results from Exp 11 page 110. This suggests that muscimol is not specific to long-term potentiation processes per se. This is important, because long-term potentation and memory share many underlying mechanisms (Izquierdo, 1994). It seems that muscimol does not fulfil all the requirements of a drug with only long-term potentiation effects. Thus, GABA may not be specifically involved in mnemonic processes but more so in fear arousal.

In vivo dialyses showed that the presentation of a conditioned fear stimulus reduced extra-cellular GABA, when compared to levels that were available during non-confrontation. This drop in extra-cellular GABA was interpreted as an effect of fear expression (Stork, Ji and Obata 2002). The addition of the $GABA_a$ agonist muscimol (increasing extra-cellular GABA) possibly negates this effect and hence the anxiolytic effect and no fear expression. Conversely, anxiogenic effects were found following the infusion of $GABA_a$ antagonists (Sanders and Shekhar 1995). Together this suggests a tonic level for extra-cellular GABA levels (Fanselow & Kim, 1992) that via pharmacological modification can disturb fear expression, potentially via alterations in

the central fear state. These findings are consistent with a role for $GABA_a$ receptors in fear arousal.

Given that the central fear state is affected by alterations in GABA tone, then it is likely that fear acquisition is also suppressed. Thus it was expected that rats do not learn the CS/US association while under the influence of muscimol because, if the central fear state is suppressed, than it is unlikely that CS/ US associations can be formed (Rodriguez-Ortiz, De la Cruz, Gutierrez, & Bermudez-Rattoni, 2005). Functional inactivation using muscimol could inhibit acquisition to context and explicit cues as measured by freezing (Helmstetter & Bellgowan, 1994; Maren & Holt, 2004; Muller, Corodimas, Fridel, & LeDoux, 1997; Wilensky, Schafe, & LeDoux, 1999, 2000) and instrumental avoidance (Poremba & Gabriel, 1999). Furthermore, muscimol could effectively prevent the acquisition of fear expressed as fear potentiated startle (van Nobelen & Kokkinidis, 2006; Wilensky, Schafe, & LeDoux, 1999). However, between 10 and 100 fold muscimol concentration was needed to suppress fear acquisition (van Nobelen & Kokkinidis, 2006). This increase was likely to be needed because of the conditioning intensity. The conditioning paradigm presented 20 CS/US trials with inter trial intervals of at least 1-3 minutes which may have increased fear levels. More muscimol was needed to suppress conditioning suggesting a cumulative effect of fear. This cumulative effect was not evident during the shock sensitization paradigm in which rats were only exposed to 10 rapid foot-shocks. Because more CS/US presentations elicit stronger conditioning (Gallistel & Gibbon, 2000), it would be interesting to determine the dose needed to suppress the effect of fewer trials. In fact, Miserendino, Sananes, Melia and Davis (1990) did just that, when investigating the effect of AP-5 on fear expression.

By reducing the number of CS/US pairing, the concentration of AP-5 could be reduced by a fourth, from 5.0µg/µl down to 1.25 µg/µl. Helmstetter and Bellgowan (1994) reported similar results with freezing behaviour, more muscimol was needed to suppress fear conditioning than fear expression.

In summary, the basolateral amygdala therefore seems to process fear arousal produced by shock and a CS, and this may be regulated by a GABAergic system (Blair, Sotres-Bayon, Moita, & LeDoux, 2005; van Nobelen & Kokkinidis, 2006).

The GABA neurotransmitter has a pentameric structure that is sensitive to benzodiazepines, barbiturates, alcohol, anaesthetics, neuro-active steroids and muscimol (Hijzen, Houtzager, Joordens, Olivier, & Slangen, 1995; Paul, 1995). Most of these agents have been shown to reduce arousal to various fear evoking stimuli (Helton, Tizzano, Monn, Schoepp, & Kallman, 1998; Tizzano, Griffey, & Schoepp, 2002). They do so via activation of the GABA receptor through increasing Cl^- ion conductance. More Cl^- channels are opened which hyperpolarize the neural membrane making it more difficult for excitatory neurotransmission to depolarize the membrane and reduce the probability of producing an action potential. The precursor for GABA is glutamate which is decarboxylated to form GABA via enzyme action (Paul, 1995). Tizzano et al (2002) showed that both GABAergic and glutamatergic systems suppressed fear expression but via different mechanisms. To illustrate that fear arousal is confined to the basolateral amygdala and is affected by GABA and not glutamate, rats were infused with NMDA and non-NMDA receptor antagonists. Thus the next system investigated was the glutamatergic system.

The function of the glutamatergic system in the basolateral amygdala

The basolateral amygdala has major innervations of excitatory systems affected by glutamate (Helton, Tizzano, Monn, Schoepp, & Kallman, 1998). Glutamate antagonism prevented basal neural functioning but also inhibited long-term potentiation (Rogan, Staubli, & LeDoux, 1997b; Weisskopf & LeDoux, 1999). A potential weakness of the shock sensitization paradigm was that shock-enhanced fear arousal may represent a form of acquired fear (Richardson, 2000; Richardson & Elsayed, 1998). However, fear acquisition is suppressed by the infusion of the NMDAr antagonist AP-5 (Goosens & Maren, 2003; Lee & Kim, 1998; van Nobelen & Kokkinidis, 2006; Walker & Davis, 2000). Thus if shock sensitization was a form of fear acquisition, than the infusion of AP-5 should suppress this. As shown in Experiment 7 a high concentration of AP-5 did not prevent the expression of the central fear state produced by foot-shock. Furthermore, AP-5 did not affect shock reactivity or startle responses. These negative results suggested that neither routine synaptic NMDA transmission nor long-term potentation functioning was involved in processing the effect of footshock, thus confirming what the behavioural studies already showed, namely that shock sensitization does not appear to involve contextual or other measurable forms of learning. Thus, NMDA was not involved in immediate fear arousal in the basolateral amygdala.

The NMDA antagonist AP-5 also has a significant role in preventing fear acquisition, similar to GABA (van Nobelen & Kokkinidis, 2006), but not through suppression of fear arousal. It does so through the prevention of the formation of the CS/US associations via inhibition of long-term potentiation (Campeau, Miserendino, & Davis, 1992; Fanselow & Kim, 1994; Fanselow, Kim, Yipp, & De Oca, 1994; Gewirtz &

Davis, 1997; Lee & Kim, 1998; Rogan, Staubli, & LeDoux, 1997b; Sigurdsson, Doyere, Cain, & LeDoux, 2007). Long-term potentiation is the result of biochemical cascades, which are necessary for structural alterations in the neurons once the formation of associations have been initiated. For example, protein kinase A and C are part of the long-term potentiation effect. Blocking these prevents fear acquisition in the basolateral amygdala (Goosens, Holt, & Maren, 2000). Investigation of some of the NMDA subunits showed that blocking the NR2B subunit prevented fear acquisition (Rodrigues, Schafe, & LeDoux, 2001), while mice lacking the NMDA receptor NR2A cannot retrieve fear associations (Moriya et al., 2000).

This failure to learn the association would also predict a failure of extinction (Falls, Miserendino, & Davis, 1992). Extinction is the acquisition of new associations between the CS and a new consequence. Usually the CS is no longer followed by the US, but by no stimulus. Thus rats learn that the predictive value of the CS has changed. These authors' rats were infused 24 hours after successful fear conditioning, but before extinction training. They did not evaluate the role of AP-5 on fear arousal and expression per se, but concluded that AP-5 prevented a NMDA-dependent learning mechanism. The shock sensitization results complement their work in eliminating the fear arousal factor. In addition, intraperitoneal injections of the NMDA partial agonist d-cycloserine have been shown to facilitate extinction (Walker, Ressler, Lu, & Davis, 2002).

NMDA is also necessary for fundamental neural communication but not necessarily involving long-term potentiation (Farb & LeDoux, 1997; Fendt, 2001; LeDoux, 2000; Weisskopf & LeDoux, 1999). However, this form of neural communication was not required during immediate fear arousal produced by footshock. To investigate if it was a

requirement during fear expression, AP-5 was infused into the basolateral amygdala before a fear expression test. In Experiment 12 rats showed significant attenuation in response to CS presentation. This was not due to ataxic effects to acoustic startle or to lack of fear prior to testing. Moreover, this was a temporary effect as rats exhibited significant levels of fear 24 h later. This was supported by the effects of AP-5 on freezing and startle behaviour to a CS and context; furthermore the blocking of the fear effect was unrelated of fear level (Fendt, 2001; Lee & Kim, 1998; Lee, Choi, Brown, & Kim, 2001; Maren, Aharonov, Stote, & Fanselow, 1996), but see (Campeau, Miserendino, & Davis, 1992; Miserendino, Sananes, Melia, & Davis, 1990). The effect of AP-5 on fear expression was unlikely to be a deficit in long-term potentiation because blocking of protein kinase A and C prior to fear expression does not attenuate this (Goosens, Holt, & Maren, 2000).

The overlap between the shock sensitization and the fear-potentiated startle paradigm showed that the AP-5 effect that occurred during the fear potentiated startle paradigm was not due to suppression of the central fear state, but possibly to a disruption of signal propagation. The general consensus is that NMDA receptors are needed not only for long-term potentiation but also for synaptic transmission within the basolateral amygdala (Farb & LeDoux, 1997; Fendt, 2001; LeDoux, 2000; Weisskopf & LeDoux, 1999).

In summary, the results of Experiments 7 and 12 showed that AP-5 infusions into the basolateral amygdala did not affect immediate fear arousal, but could prevent fear expression to a CS. This outcome was not due to rats failing to process fear arousal, but

more likely due to higher cognitive functioning that was inhibited via NMDA signal propagation.

The effect of the AMPA receptor antagonist CNQX was also evaluated during both paradigms. AMPA sensitive neurons are involved in the regulation of the release of neurotransmitters in several brain areas including the basolateral amygdala (Vizi, 2003). AMPA receptors also need to be stimulated before long-term potentiation can be induced (Malinow, Mainen, & Hayashi, 2000; Reymann & Frey, 2007). Moreover, as AMPA receptors also require activation for the expression of long-term potentiation (Nicoll, 2003), they exhibit a similar dual role to NMDA receptors, long-term potentiation and normal excitatory neural transmission. Experiment 7 showed that the AMPAr antagonist, CNQX had no measurable effect on fear arousal produced by footshock. No significant effects on shock reactivity or startle were noticed. These results indicate that AMPA receptors used in normal synaptic transmission were not required during fear arousal produced by footshock. This also indicates that the shock sensitization paradigm did not involve a long-term potentiation dependent process because long-term potentiation requires AMPA receptor activation as part of this development. For example, fear acquisition was prevented after blocking the AMPA receptors via CNQX infusion prior to conditioning (McKernan & Shinnick-Gallagher, 1997; Pistell & Falls, 2008). Conversely, stimulation of AMPA receptors accelerates fear acquisition (Rogan, Staubli, & LeDoux, 1997a). Once fear has been acquired and consolidated it can be prevented from being expressed by inhibiting the AMPA receptor. Results reported in Experiment 13 showed that CNQX could significantly attenuate fear expression to a CS, similar to

results reported by others (Bianchin et al., 1993; Kim, Campeau, Falls, & Davis, 1993). CNQX also prevented the expression of conditioned taste aversion (Yasoshima, Morimoto, & Yamamoto, 2000) and inhibitory avoidance expression (Bianchin et al., 1993; Izquierdo et al., 1993; Roesler et al., 1999).

The effects of CNQX on CS-induced fear expression was not due to inhibition of the fear arousal system, because the shock sensitization paradigm clearly showed that CNQX could not reduce immediate fear arousal. Thus, during fear expression to a CS, the effect of CNQX on AMPA receptors suggests an inhibition of excitatory neurotransmission rather than suppression of fear arousal (Li, Stutzmann, & LeDoux, 1996; Mamou, Gamache, & Nader, 2006). These effects were most likely due to a deficit in neural functioning that is involved in long-term potentiation. During this process AMPA receptors increase in number and sensitivity (Malinow, Mainen, & Hayashi, 2000; Maren, Tocco, Standley, Baudry, & Thompson, 1993). Thus during the expression of long-term potentiation it is highly likely that CNQX prevents these AMPA receptors from functioning and thus the expression of fear during CS presentation.

In summary, CNQX does not affect a central fear state but can prevent fear expression probably via inhibition of the expression of long-term potentiation by blocking the AMPA receptor.

The effects of anisomycin in the basolateral amygdala

The effects of anisomycin were evaluated because in the basolateral amygdala a CS-US association takes place which is long-term potentiation dependent (Rogan, Staubli, & LeDoux, 1997b). The development of this process involves protein synthesis

that implements the structural changes that are characteristic of long-term potentiation (Reymann & Frey, 2007). The infusion of anisomycin prevents the structural changes from occurring when infused prior to fear acquisition (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999). Thus, if immediate fear arousal is an expression of a learned phenomenon (Richardson, 2000; Richardson & Elsayed, 1998), then anisomycin should prevent long-term potentiation and thus the expression of immediate fear arousal. As was clearly demonstrated in Experiment 8, anisomycin had no significant effect during the shock sensitization paradigm. This supports the likelihood that immediate fear arousal produced by footshock is an unlearned fear response and not memory formation dependent.

Fear expression can be a long-term potentiation dependent process and is sensitive to disruption of protein synthesis. In Experiment 14 it was demonstrated that anisomycin could significantly affect fear expression 24 hours post infusion. Rats that expressed fear under the influence of anisomycin were unable to express this 24 hours later. The results support findings of Nader, Schafe, and LeDoux, (2000a) and Mamou, Gamache, and Nader (2006). Some notable differences between these authors' paradigm and the current paradigm were that rats in the current paradigm were infused prior to testing rather than directly after testing, and fear was tested via startle as opposed to freezing. In addition, the number of conditioning trials (35) in the current research was significantly more than that of Nader et al. (2000) who used one tone paired with one footshock. Infusion of anisomycin directly before or after one-trial fear conditioning could prevent the expression of freezing behaviour 24 hours later (Mamou, Gamache, & Nader, 2006; Schafe & LeDoux, 2000; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999). It has been

suggested that the retrieval of memories makes them labile, thus making them susceptible to change. These changes can be inhibited by the infusion of a protein synthesis inhibitor (Mamou, Gamache, & Nader, 2006). Moreover, memories need to be directly activated by the CS to become susceptible to the adverse effects of anisomycin (Debiec, Doyere, Nader, & LeDoux, 2006). This effect becomes less pronounced as time passes (Milekic & Alberini, 2002).

In the current paradigm, rats were tested only 24 hours after infusion during which no fear was shown. It would have been interesting to see if this deficit was measurable at various later times. Mice that have been conditioned with a single context / footshock exposure, and infused with anisomycin after retrieval showed a freezing behaviour deficit 24 h later but this deficit attenuated over time. By the time 21 days were reached the deficit had disappeared (Lattel & Abel, 2004).

In summary, protein synthesis was not involved in immediate fear arousal produced by footshock, but was implicated in fear expression to a CS. The specific effect was noticed 24 hours later when rats were unable to express fear. This effect was unlikely to be due to a fear arousal deficit.

The function of the dopaminergic system in the basolateral amygdala.

D1 agonist SKF 38393

The last major neurotransmitter group investigated was dopamine, which primarily acts on D1 and D2 receptor sub types. D1 and D2 function via different mechanisms (Missale, Nash, Robinson, Jaber, & Caron, 1998), and manipulation of these in the ventral tegmental area can influence fear processing in the amygdala (Borowski & Kokkinidis, 1996; Munro & Kokkinidis, 1997). Both D1 and D2 receptors are found in

abundance in the basolateral amygdala (Boysen, McGonigle, & Molinoff, 1986; Leonard et al., 2003) and the intercalated islands between the central and basolateral amygdala (Fuxe et al., 2003) which have been implicated in fear acquisition and memory (Boysen, McGonigle, & Molinoff, 1986; Missale, Nash, Robinson, Jaber, & Caron, 1998). Although D1 receptor antagonists have been investigated to understand the receptors role in fear processing, relatively little is known about the effects of D1 agonists.

The D1 agonist SKF 38393 was infused into the basolateral amygdala to investigate the function of D1 receptors on fear arousal by footshock, and to compare this result to fear arousal produced by a CS. The effect of the D1 agonist on immediate fear arousal was marred by the high reactivity of the rats to acoustic startle after infusion. Since startle reactivity after footshock did not decline and was similar to the saline control group, it was concluded that D1 receptors did not affect immediate fear arousal produced by footshock. Similar results have been found after chronic cocaine exposure during the shock sensitization paradigm (Willick & Kokkinidis, 1995). Moreover, the D1 antagonist SCH 23390 was also not involved in immediate fear arousal produced by footshock (Greba & Kokkinidis, 2000). Together these results suggest that dopamine has little effect on immediate fear arousal.

These results are consistent with the findings that dopamine regulates the formation and expression of conditioned fear (Greba & Kokkinidis, 2000; Inoei, Izumi, Maki, Muraki, & Koyama, 2000; Pezze & Feldon, 2004), but not immediate fear arousal. Specifically, the induction of long-term potentiation is gated by dopamine (Bissiere, Humeau, & Luthi, 2003; Loretan, Bissiere, & Luthi, 2004). Together these results imply a more specific function for dopamine in memory formation (Kerr & Wickens, 2001). As

was shown earlier, neither the long-term potentiation-attenuating NMDA nor non-NMDA receptor antagonists were involved in immediate fear arousal produced by footshock. Since the induction of a central fear state by footshock does not appear to involve the development of an association, and thus memory formation, it was unlikely that dopamine affected the central fear state.

However, as shown in Experiment 15 the expression of a central fear state aroused by CS presentation was completely inhibited by the infusion of the D1 agonist SKF 38393. This was a new finding, and complementary to the research that showed that the D1 antagonist SCH 23390 could also prevent fear expression (Waddington Lamont & Kokkinidis, 1998). However, these authors infused SCH 23390 into the whole amygdala and this may have confounded the outcome since the basolateral and central amygdala have different functions in fear processing (Zimmerman, Rabinak, McLachlan, & Maren, 2007).

Thus, both the D1 agonist and antagonist had no influence on immediate fear arousal but both do prevent fear expression to a CS. Dopamine has been shown to excite basolateral interneurons thereby changing the contribution of amygdaloid function to behaviour (Kroner, Rosenkranz, Grace, & Barrionuevo, 2005). In general, dopamine agonists inhibit dopamine release. However, the method of action is dependent upon the neuronal feedback loops involved (Roth & Elsworth, 1995). These are controlled by dopamine autoreceptors and postsynaptic dopamine receptors. Furthermore, the efficiency and the nature of the afferent inputs mediate the outcome of the agonistic action (Roth & Elsworth, 1995). Thus, actions of D1 agonists and antagonists may depend upon the properties of the local amygdaloid neurons.

This new finding adds to the existing knowledge that not only dopamine antagonism but also agonism is involved in fear expression thus suggesting a role for modulation, and implying that if dopamine homeostasis is unbalanced by either agonism or antagonism, fear expression ceases. This does not appear to involve inhibition of immediate fear arousal but possibly does involve a cognitive process (Waddington Lamont & Kokkinidis, 1998).

D2 agonist quinpirole

The results of Experiments 9 and 16 indicated a role for the D2 agonist, quinpirole in fear expression to CS presentation but not in immediate fear arousal. In Experiment 9 rats were more active after drug infusion but this did not affect overall immediate fear arousal to footshock. Additionally, the D2 agonist could prevent fear expression to a CS but the results from Experiment 9 suggest this was not mediated by a suppression of immediate fear arousal. Similar results have been reported using the D2 antagonist, raclopride (Greba, Gifkins, & Kokkinidis, 2001). The D2 antagonist did not affect immediate fear arousal by footshock but did attenuate fear acquisition. This was reported to be due to a failure in long-term potentiation initiation. In vivo intracellular recordings have shown that indeed plasticity is affected by dopamine antagonist haloperidol via a reduction in post synaptic potentials (Rosenkranz & Grace, 2002).

Systemic injections have shown that quinpirole impairs recall of fear memories (Nader & LeDoux, 1999a). This effect was due to a failing retrieval mechanism in the US/CS association. Similar effects have been shown using mice in which quinpirole blocked extinction but the D2 antagonist, sulpride, facilitated this effect (Ponnusamy,

Nissim, & Barad, 2005). Thus the effect produced by quinpirole during CS evoked fear expression was more likely to have been due to recall failure of the CS/US association than to a deficit in immediate fear arousal.

Overall, the effects of dopamine agonists on fear processing were due to failures in recall of the fear association, which were perhaps mediated through a long-term potentiated dependent mechanism.

Summary of findings in the basolateral amygdala

The major finding in this section was that small doses of muscimol prevented fear expression to a US (van Nobelen & Kokkinidis, 2006) and to a CS. The novelty of this was that by comparing these two paradigms it was possible to investigate the role of various neurotransmitter groups during the expression of US- and CS-induced central fear states. Of the three major neurotransmitter systems investigated, only the GABAergic system mediated fear expression to both US and CS fear eliciting stimuli. This finding indicates a unique function for the GABAergic inhibitory system in the basolateral amygdala and unlike the glutamatergic and dopaminergic systems which were not involved in immediate fear arousal but could prevent fear expression to a CS.

By using the shock sensitization paradigm it became possible to evaluate the three components elicited by footshock, namely reflex, pain, and emotion. The results from Experiments 6 to 16 indicate that the basolateral amygdala processed the emotive component via a GABAergic system. There is clear evidence in the literature that the basolateral amygdala indeed processes the association between the US/CS (Romanski, Clugnet, Bordi, & LeDoux, 1993). But it was unclear which component of the US was associated here. Experiments 6 to 16 indicate this is the emotive component. The

basolateral amygdala receives afferents from areas that receive external stimuli such as footshock, auditory and visual inputs. Lately it has become clear that not only the basolateral amygdala receives these inputs but also that the central amygdala is receptive to these (Pare, Quirk, & LeDoux, 2004; Pascoe & Kapp, 1985). Would the central amygdala also process the emotive value of the footshock or could it be involved in processing reflex or pain?

Comparisons between central fear states produced by foot-shock and CS presentation in the central amygdala

The role of the central amygdala was investigated because a large array of literature suggested that this is an area responsible for processing pain (Almeida, Roizenblatt, & Tufik, 2004; Crown, King, Meagher, & Grau, 2000; Neugebauer, 2007), and the execution of fear behaviours (LeDoux, Iwata, Cicchetti, & Reis, 1988).

Footshock had been shown to produce pain (Crown, King, Meagher, & Grau, 2000) and also immediate fear arousal (Davis, 1989; van Nobelen & Kokkinidis, 2006), but the role of neurotransmitters in the central amygdala using the shock sensitization paradigm have not been investigated. Although lesions to the central amygdala will reduce the shock sensitization effect, no specific reasons have been suggested for why this happens (Hitchcock, Sananes, & Davis, 1989). In addition, lesions block the enhancement of vocalization after shock (Crown, King, Meagher, & Grau, 2000). The increase in vocalization after shock is a measure of hyperalgesia (enhanced pain). Thus, lesions of the central amygdala reduce pain, at least as measured by vocalization.

The following section compared the data collected during the shock sensitization paradigm to the data collected during the fear potentiated startle paradigm in the central amygdala. Three major neurotransmitters were investigated, namely GABA, glutamate, and dopamine. Protein synthesis was also evaluated because there is evidence that long-term potentiation does occur in the central amygdala (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

The function of the glutamatergic system in the central amygdala.

The central amygdala is part of the nociceptive circuit (Manning, 1998; Manning, Martin, & Meng, 2003; Neugebauer & Li, 2003; Neugebauer, Li, Bird, & Han, 2004) and thus may receive pain inputs caused by foot-shock. Abundant nociceptive information from the spinal cord arrives via the parabrachial nucleus to the central amygdala (Almeida, Roizenblatt, & Tufik, 2004; Samson, Duvarci, & Pare, 2005). This pathway is dependent upon glutamatergic processes involving both NMDA and AMPA receptors (de Armentia & Sah, 2007). Noxious stimulation can induce synaptic plasticity of which the underlying mechanism is very similar to long-term potentiation (Neugebauer & Li, 2003). Both processes involve the activation of AMPA and NMDA receptors (Ji, Kohno, Moore, & Woolf, 2003). Moreover, maintenance of those processes is mostly regulated by similar biochemical cascades (Ji & Woolf, 2001).

Extra cellular recordings have shown that the central amygdala is receptive to noxious and non-noxious information arising from the parabrachial area (Neugebauer & Li, 2002). Whole cell patch clamp recordings indicated a significant increase in synaptic transmission in the parabrachial-central amygdala synapses in rats with visceral pain (Han & Neugebauer, 2004). The basolateral amygdala receives polymodal sensory information from other areas which are directed towards the central amygdala but does not appear to process pain information. For example, whole cell patch clamp recordings did not register increases in synaptic transmission during visceral pain in basolateral-central amygdala synapses (Han & Neugebauer, 2004). This suggests that pain information may not pass through the basolateral amygdala but is directly processed by the central amygdala.

A significant finding, as shown in Experiments 17 and 18, concerning the central amygdala was that glutamate affected the shock sensitization paradigm. Both the NMDA and the non-NMDA receptor antagonist could prevent the expression of shock enhanced startle. The NMDAr antagonist AP-5 could reliably suppress shock enhanced startle in rats under influence of high concentrations (5μg/μl) but also low concentrations (0.6μg/μl). This deficit was not due to suppression of footshock reactivity or startle. No significant differences in startle responses were noted between the control and experimental groups. In addition, footshock reactivity was not significantly reduced. Likewise, footshock is not affected by functional inactivation of this area (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

Shock produces motor-reflexes, pain and emotion (Borszcz, 1993, 1995; Crown, King, Meagher, & Grau, 2000). The motor-reflex was not affected during the shock sensitization paradigm in either the basolateral or the central amygdala. Therefore, it is probable that the central amygdala is involved in processing the pain or the emotive component of shock. Since the emotive element is suggested to be controlled by the GABAergic system in the BLA (Blair, Sotres-Bayon, Moita, & LeDoux, 2005; Rorick & Steinmetz, 2005; van Nobelen & Kokkinidis, 2006), it is highly likely that the central amygdala processes the pain component produced by shock.

This interpretation is supported by results reported by Rorick and Steinmetz (2005) who indicated that increased excitatory neural activity in the central amygdala was related to intensity of shock. The magnitude of the neural response was related to the strength of the behavioural response. While lesions of the central amygdala prevented rats from exhibiting vocal after discharges, which are indices of pain (Borszcz & Leaton,

2003), these ultra sonic vocalizations were reduced after morphine injections (Oliveira & Barros, 2006). Morphine can significantly reduce nociception after formalin injections but this effect was abolished after central lesions but not basolateral amygdala lesions (Manning, 1998). Similar effects have been found by cannabinoid induced anti-nociception (Manning, Martin, & Meng, 2003), suggesting that the central amygdala is part of the nociceptive circuit, but that the basolateral amygdala is not.

Evidence that the AP-5-induced shock deficit is produced by a reduction in pain processing came from research conducted (amongst others) by Li and Neugebauer (2004; Neugebauer & Li, 2002). Extra cellular recordings show that a vast majority of central amygdaloid cells respond to brief noxious stimulation. They investigated these cells and have shown that two major subtypes are responsive to pain and exhibit plasticity, namely multi-receptive (MR) and non-responsive neurons (Li & Neugebauer, 2004; Neugebauer & Li, 2003). MR neurons respond to both noxious and non-noxious stimuli and are glutamate sensitive (Li & Neugebauer, 2004). For example, MR neurons respond to touch but more so to pinching and pressing. Touching does not produce a withdrawal reflex whereas pinching does. Infusion of AP-5 inhibits MR neurons from firing under noxious but not non-noxious stimulation. Similar results have been reported in an arthritic pain model in which NMDAr antagonism prevented pain but not normal synaptic transmission (Bird et al., 2005). This indicates that AP-5 prevents transmission of potentially harmful but not harmless information. Similarly, the NMDAr antagonist, ketamine, prevented pain produced by electrical stimulation, but did not affect light tactile stimulation (Klein et al., 2007).

In the central amygdala there can be synaptic plasticity and this may involve an long-term potentiation like mechanism (Woolf & Salter, 2000). One of the mediators of this process is NMDA. The shock sensitization deficit produced by AP-5 is unlikely to long-term potentiation dependent because it has been shown that the shock sensitization paradigm does not involve measurable formations of fear associations (see Experiments 1-10 and van Nobelen and Kokkinidis, 2006).

The dose response study showed that NMDA receptors played a specific role in suppressing shock sensitization. If this was due to a suppression of the central fear state it was expected that AP-5 would also reduce fear expression to the CS especially since the central amygdala is necessary for the expression of fear-potentiated startle (Fendt & Fanselow, 1999). Conversely, if AP-5 prevented the transmission of noxious but not non-noxious information then AP-5 should not affect fear expression.

Experiment 22 clearly demonstrated that the infusion of AP-5 prior to CS presentation did not affect fear expression. This was not due to a lack of efficacy of the drug, or deficiencies of the paradigm since AP-5 was effective in preventing fear expression in the basolateral amygdala. A possible explanation for the different responses to the two paradigms was that testing for fear using a conditioned stimulus does not involve pain. Thus, recall of the meaning of the CS will entail activation of the central fear state but not the physical properties of pain that were associated with footshock. This is analogous to AP-5 inhibiting neuronal firing during noxious stimuli (footshock) and not during non-noxious stimuli (CS presentation).

Moreover, animals can exhibit conditioned pain reduction, whereby formalin-induced pain is suppressed in a context that has previously been paired with shock.

Lesioning the central amygdala reduced this conditioned pain suppression and rats showed significant pain behaviour during the formalin test (Helmstetter, 1992) thereby indicating that, during CS presentation, rats can become less sensitive to external pain. This also implies that AP-5 has even less effect because of the conditioned pain reduction already processed in the central amygdala.

If AP-5 does suppress nociception during footshock it would be expected that learning the CS-US association would be inhibited because AP-5 would prevent pain and thus hinder the CS/US association from forming. This was precisely what Goosen and Maren (2003) found AP-5 prevented fear acquisition, but more importantly AP-5 did not prevent 'savings' of conditioned fear in the central amygdala. Fear saving is a measure of fear acquisition that may not be noticed during initial fear testing but becomes apparent during further fear acquisition trials. Rats that show savings learn CS/US associations faster than the control group to both context and explicit tone cues, because residual (partial) memory exists. In their study rats were infused with AP-5 prior to conditioning and showed no fear 24 h later. Then they received 1 CS/US trial on day 1, 2, 3 and 4. Freezing behaviour was recorded continuously. The rats with AP-5 infused into the central amygdala showed faster acquisition than the control groups. On the other hand, rats with basolateral amygdala cannulae did not show acquisition OR savings. It was postulated that the basolateral amygdala deficit was due to AP-5 preventing the CS-US association via a long-term potentiation mechanism, and thus preventing fear acquisition and savings. Conversely, it was also suggested that the central amygdala deficit did not involve long-term potentiation because savings were made indicating that learning did occur albeit attenuated.

An alternative explanation for the results found by Goosens and Maren (2003) could be that AP-5 blocked pain during conditioning and thus prevented a CS/US association from forming. But since footshock also elicits an emotive state that is processed in the basolateral amygdala, a CS/US association could have been formed here that later appeared as a 'saving' (Goosens & Maren, 2003). Other research showed that lesions of the central amygdala prevented fear acquisition, but not reacquisition (Kim & Davis, 1993; Zimmerman, Rabinak, McLachlan, & Maren, 2007). These authors also proposed a dual system whereby savings were stored in the basolateral amygdala. These saving effects were not found in the basolateral amygdala after functional inactivation (Maren, Yap, & Goosens, 2001). Thus this inhibitory effect of AP-5 on fear acquisition may not involve long-term potentiation but perhaps a nociceptive effect.

Even though AP-5 is associated with memory formation and the infusion of this drug prevents the NMDA receptors from functioning and therefore inhibiting initiation of this process, most long-term potentiation induction relies upon activation of protein kinase A and C (Huang & Kandel, 1998). Infusion of H7 prevents this activation and thus the formation of long-term potentiation. But the infusion of H7 into the central amygdala does not prevent fear acquisition (Goosens, Holt, & Maren, 2000). This would indicate that NMDA-dependent long-term potentiation is not taking place in the central amygdala and that the AP-5 effect is a reduction in pain processing. Conversely, H7 significantly prevented fear acquisition to tone and context when infused into the basolateral amygdala, indicating that this area is sensitive to disruption of long-term potentiation (Goosens, Holt, & Maren, 2000).

On further investigation, H7 prevented both PKA and PKC from acting and in combination had no effect on fear acquisition. However, PKA but not PKC is involved in pain related synaptic plasticity. For example, AP-5 has no effect on synaptic transmission in the central amygdala in normal rats but can significantly attenuate neural transmission under arthritic conditions. Arthritic conditions are instigated after kaolin injections into the knee joint. Six to eight hours later pain-related synaptic plasticity was evident in the central amygdala which was PKA dependent (Bird et al., 2005). Likewise, audible and ultra sonic vocalizations were reduced after micro-dialyses with PKA but not PKC inhibitors in rats with chronic arthritis. This was interpreted as PKA increasing NMDA receptor functioning through independent signalling pathways which were different from hippocampal long-term potentiation (Fu et al., 2008). These results indicate that at least two types of long-term potentiation exist, of which one is involved in pain plasticity and the other is not (Ji, Kohno, Moore, & Woolf, 2003; Ji & Woolf, 2001).

NMDA neurotransmission has a prominent role in pain reduction in the central amygdala but also in afferent projections towards the central amygdala. Infusion of AP-5 at a spinal level reduced pain in rats with acute pancreatitis (Zhang, Zhang, & Westlund, 2004). The periaqueductal gray and the rostral ventromedial medulla systems mediate analgesia but also enhanced nociception, AP-5 significantly attenuates pain enhancement after noxious stimulation (Xu, Kim, Neubert, & Heinricher, 2007). Both the central and basolateral amygdala receive footshock information from cortical and sub-cortical structures. Nociceptive information to the central amygdala from the parabrachial tract is glutamate and corticotrophin releasing factor sensitive (Ji & Neugebauer, 2007). Cutaneous and intraplantar application of glutamate antagonists significantly reduced

formalin-induced nociceptive behaviours (Davidson & Carlton, 1998; Davidson, Coggeshall, & Carlton, 1997).

Based on the information that the NMDA receptor affects pain perception, the AMPA receptor was also investigated with both paradigms. As noted earlier, the glutamate antagonist AP-5 prevented fear arousal produced by footshock. A similar effect was found for CNQX. As shown in Experiment 17 the non-NMDA antagonist CNQX could effectively block the shock sensitization effect, but did not attenuate shock reactivity or startle. This deficit was accounted for by a reduction in pain processing and not in general neural processes.

If it was a deficit in general neural processing than it was expected that other paradigms involving fear processing would also be affected after AMPA antagonism. For example, Walker and Davis (1997b) tested rats in the light enhanced startle paradigm. Very bright lights are aversive and increase the startle response. But the infusion of an AMPA antagonist (NBQX) did not reduce enhanced startle. Similarly, the shock sensitization paradigm can measure immediate fear arousal via increased post-shock startle. Both paradigms are aversive, but only the shock sensitization paradigm was affected by an AMPA antagonist. The main difference was that the shock sensitization paradigm used shock to induce fear whereas the light enhanced startle paradigm used light. Shock produces pain, light does not.

If AMPA neurotransmission prevents pain processing then CNQX should not influence fear expression to CS presentation. This was not found. Rats infused with CNQX failed to show enhanced startle to CS presentation. These results are supported by findings of Walker and Davis 1997, who infused rats with NBQX prior to fear testing and

noted that the animals failed to exhibit potentiated fear. Thus, the AMPA antagonist CNQX blocked immediate fear arousal and also fear expression during CS presentations. This dual function of AMPA receptors has also been noticed by other (Li & Neugebauer, 2004; Walker & Davis, 1997b). For example, Li and Neugebauer (2004) reported that CNQX interferes with neural firing patterns associated with pain perception, but also with processing of non-noxious stimuli. This suggested a dual role for CNQX in pain and non-painful information processing which was difficult to prise apart with these two paradigms. Similar results have been reported in spinal cord research, in which non-NMDA antagonists prevented the action of both noxious and non-noxious stimuli while NMDA antagonists only did so for noxious stimuli (King & Lopez-Garcia, 1993).

It was suggested that non-NMDA is involved in faster and perhaps earlier synaptic transmission than NMDA (Imamachi, Saito, Hara, Sakura, & Kosaka, 1999; King & Lopez-Garcia, 1993). This fast non-NMDA regulated excitatory synaptic transmission appears to be triggered before NMDA receptors do. Hence non-NMDA antagonists block both noxious and non-noxious stimuli but NMDA antagonists only block noxious stimuli. Both the cortical and thalamic pathways carry US information to the amygdala. During routine neural transmission non-NMDA receptor antagonsim prevented transmission in both thalamo-amygdala and cortico-amygdala pathways while NMDA receptors had a lesser role (Li, Stutzmann, & LeDoux, 1996), again indicating differences in receptor specificity.

Because AMPA antagonism could prevent the shock sensitization effect it was expected that a CS/US association would also be inhibited. AP-5 and CNQX infused before fear acquisition trials prevented fear expression (Pistell & Falls, 2008). Walker

and Davis (2002) reported that NBQX (AMPA antagonist) could effectively prevent CS/US associations from forming, as expressed by fear-potentiated startle. They suggested that this was most likely due to a learning impairment, but did not exclude alternative output pathways that may provide the basolateral amygdala with CS/US information. An alternative explanation might be that the rats were not able to experience pain and thus not form an association between the CS and US. The underlying mechanism is most likely similar to that reported in the pain literature, in that non-NMDA receptors are involved in pain related plasticity as well as in normal neurotransmission (Li and Neugebauer 2004). The excitatory role of glutamate appears to have an important role in the central amygdala as well in pathways closer to the nociceptive source.

Both NMDA and the AMPA receptors appear to be involved in pain perception. Furthermore, using knock-out mice, the kainate receptor (another non-NMDA receptor) is also involved in pain, particularly in chemical/inflammatory pain (Baranaukas & Nistri, 1998). Overall these results indicate a significant role for glutamate receptors in pain perception in the central amygdala.

The effect of anisomycin in the central amygdala.

The function of the central amygdala is not only in the execution of autonomic functions (Ko, Zhao, Toyoda, Qui, & Zhuo, 2005) but also in CS/US association formation and expression (Davis, 1986; LeDoux, 2000). Thus it was important to investigate the role of protein synthesis via the synthesis inhibitor, anisomycin. The infusion of anisomycin prior to conditioned taste aversion prevented its acquisition (Wilensky, Schafe, Kristensen, & LeDoux, 2006). In addition, the infusion of anisomycin

after conditioning affected long-term memory formation as rats were unable to express freezing behaviour 24 hours later (Bahar, Samuel, Hazvi, & Dudai, 2003). The formation of CS/US associations and memories involves the induction of phosphorylation of CREB (the addition of a phosphate group) which is part of long term potentiation. It has been shown that animals retrieving cued fear associations produce increased CREB phosphorylation in the central amygdala (Wilensky, Schafe, Kristensen, & LeDoux, 2006). Thus there is evidence that anisomycin can affect fear processing in the central amygdala.

In Experiment 20 the shock sensitization paradigm showed that anisomycin was not effective in suppressing the central fear state produced by footshock. Even though rats were agitated in the post-drug group, they still showed increased post-shock startle that was of a similar level to the saline group. The effect of anisomycin on fear expression to a CS was investigated in Experiment 24. Again the rats showed high agitation to acoustic startle probes while under the drug influence, but this was not enhanced by CS presentation. Since the startle responses to the CS were of a similar magnitude to the saline group, it was concluded that anisomycin may have no significant effect on CS presentation. However, because of the ambiguity of the results, this experiment would benefit from repetition. Unlike the effect in the basolateral amygdala whereby anisomycin prevented fear expression 24 hours later, infusion into the central amygdala appears to be ineffective. These negative results indicate that during fear expression to both footshock and CS, protein synthesis may not be essential. Moreover, it shows that pain expression during footshock is not affected by protein synthesis inhibition.

The function of the GABAergic system in the central amygdala.

The role of GABAergic activity on pain processing in the central amygdala has not received much attention. Even though the central amygdala has a well developed GABAergic system (Hall, Thomas, & Everitt, 2001), this was, as shown in Experiments 19 and 25, not actively involved in either fear arousal to footshock or to a CS. Thus unlike the basolateral amygdala, a low concentration of muscimol infused into the central amygdala had no effect on fear expression. However, larger concentrations that inactivate the central amygdala could prevent fear acquisition and expression (Cassell, Freedman, & Shi, 1999; Delaney & Sah, 1999; Zimmerman, Rabinak, McLachlan, & Maren, 2007). Furthermore, the infusion of the GABA antagonist bicuculline methiodide does not affect fear conditioning (Burhans & Schreurs, 2008; Holahan & White, 2004; Wilensky, Schafe, Kristensen, & LeDoux, 2006). This indicates that anaesthetic effects of high concentration of muscimol were responsible for the deficits in fear acquisition and expression.

Research on pain perception in the central amygdala has also shown that higher doses of muscimol (0.025 μg/μl) but not lower (0.01μg/μl) affected extra pain inflicted onto an injured hind paw (Pedersen, Scheel-Kruger, & Blackburn-Munro, 2007). The high dose rate suggests a local anaesthetic but not a specific GABAergic effect because the lower dose did not attenuate pain. If a GABAergic system was responsible for pain attenuation, then a small dose should also have been effective. This indicates that GABA neurotransmission is not involved in actual pain perception processing in the central amygdala (Pedersen, Scheel-Kruger, & Blackburn-Munro, 2007). However, anti-nociception can be achieved in the spinal cord using benzodiazepines, which affect

certain GABAergic receptors that contain α2 and α3 subunits (Gao, ren, Zhang, & Zhao, 2004; Tanimoto, Nakagawa, Yamauchi, Minami, & Satoh, 2003), but this has not been investigated in the central amygdala yet.

Findings reported by Holahan and White (2004) showed that inactivation of the central amygdala via high doses of muscimol (0.05μg/μl) could prevent freezing behaviour during 1 minute inter-shock intervals. These rats exhibited similar behaviour to the non-shocked saline group. Conversely, the saline-shocked group showed significant freezing behaviour during the inter-shock intervals. This indicated that inactivation of the centrala amygdala prevented the expression of freezing behaviour. Moreover, twenty four hours later, during testing for contextual freezing behaviour, the muscimol group showed an increase in freezing behaviour as compared to the non-shocked group. This suggests that inactivation of the central amygdala during shock does not prevent learning of the context-shock association. Even though freezing behaviour was significantly attenuated during shock, some context-shock association had clearly formed. This effect was interpreted as a failure of mnemonic functioning (Knalb et al., 2008).

An alternative explanation could be that muscimol inactivation of the central amygdala may prevent pain and consequently the expression of freezing behaviour during shock exposure. But, because the rats still expressed some freezing behaviour during testing it is possible that suppression of pain was not sufficient to prevent contextual fear conditioning. For example, rabbits infused with muscimol into the central amygdala showed a significant attenuation in the development of conditioned responses. But over time these responses did develop (Pedersen, Scheel-Kruger, & Blackburn-Munro, 2007) thereby suggesting that conditioning did occur but that inactivation of the

central amygdala attenuated this. Footshock produced respsonses are carried via the thalamic and cortical pathways to both the central and basolateral amygdala (Burhans & Schreurs, 2008), of which the basolateral amygdala is responsible for the formation of an association between the CS and US (Romanski, Clugnet, Bordi, & LeDoux, 1993). Thus it is highly likely that some US/CS information is retained in the basolateral amygdala which is expressed during later testing (Goosens & Maren, 2003; Zimmerman, Rabinak, McLachlan, & Maren, 2007).

Overall, the results show that a low dose of muscimol had no significant effect on footshock-enhanced startle, indicating that pain was not inhibited by a GABAergic system. Moreover a GABAergic system was not required for fear expression to an explict cue.

The function of the dopaminergic system in the central amygdala.

D1 agonist SKF 38393

Heavy dopaminergic innervations are found in the central amygdala (Pare, Quirk, & LeDoux, 2004), but their function in fear arousal produced by footshock or by a CS has not received much attention. Limbic areas such as the periaqueductal gray and the nucleus accumbens are sensitive to dopaminergic disruption and interfere with nociception (Boysen, McGonigle, & Molinoff, 1986; Cassell, Freedman, & Shi, 1999; Kilts, Anderson, Ely, & Nishita, 1987; Young & Rees, 1998). The role of D1 and D2 agonists in pain perception in the central amygdala is not yet understood.

Even though the central amygdala has abundant dopamine innervations and extra-cellular dopamine is significantly augmented after footshock (Flores, Banoua, Galan-Rodriguez, & Fernandez-Espejo, 2004; Taylor, Joshi, & Uppal, 2003) the D1 agonist,

SKF 38393, had little effect on shock-enhanced acoustic startle. As reported in Experiment 21 no suppression of acoustic startle was seen after footshock, furthermore, footshock reactivity or startle was not affected. Thus, the D1 agonist SKF 38393 did not affect pain processing in the central amygdala. Surprisingly, as was shown in Experiment 26, under the influence of SKF 38393 fear expression to a conditioned stimulus was significantly increased.

The combined results of the shock sensitization and fear-potentiated startle paradigms suggest that the D1 agonist failed to affect a central fear state per se. Fear levels as measured by the acoustic startle reflex during the shock sensitization paradigm were not augmented compared to saline. Thus, the infusion of SKF 38393 did not generate more fear as compared to the saline group. However, fear expression during conditioned stimulus exposure was significantly enhanced. A possible reason for this was that SKF 38393 enhanced fear expression via a sharply attuned cognitive system.

Thus, the presentation of the CS may trigger attention and the dopamine D1 agonist may improve concentration by excluding other external stimuli and thus enhancing the fear potentiated startle effect. Intraperitoneal injections of cocaine, amphetamine or SKF 38393 all prevented fear extinction, and could restore extinguished fear at a later date (Young & Rees, 1998). Memory retrieval can be enhanced by amphetamine injections (Borowski & Kokkinidis, 1998; Willick & Kokkinidis, 1995). It is suggested that cocaine and SKF 38393 have similar memory-enhancing properties, probably through associative processes involved in the recall of conditioned fear (Quartermain & Judge, 1983).

On the other hand, research investigating the effect of SKF 38393 on contextual-fear expression 24 hours after a single footshock failed to elicit enhanced fear expression

(Borowski & Kokkinidis, 1998). However, recall of contextual cues involves different neural circuits from recall of specific cues (Selden, Everitt, Jarrard, & Robbins, 1991). Moreover, a single conditioning trial may not produce conditioning of sufficient strength and thus not evoke such strong fear memories.

It has been shown that the central amygdala has a role in orienting responses to light when paired with tone and food (Inoei, Izumi, Maki, Muraki, & Koyama, 2000), especially alterations in the consequences of the CS. Thus, during conditioning the CS predicts shock while during testing the CS is followed by acoustic probes. With humans it was found that signals predicting pain strongly engaged attention, while signals predicting safety did not (Gallagher & Holland, 1994; McDannald, Kerfoot, Gallagher, & Holland, 2005) Moreover, attentional functioning in the medial prefrontal cortex is significantly increased by the infusion of SKF 38393 since rats show increased accuracy and speed in cognitive functioning (Van Damme et al., 2004). The medial prefrontal cortex has connections with the central amygdala and is involved in appetitive conditioning and drug addiction.

The role of dopamine D1 receptors in pain processing is limited. The D1 antagonist, SCH 23390, does not affect pain-induced vocalization (Granon et al., 2000). Mice lacking the D1 receptor also exhibit normal pain behaviours (Inoei, Izumi, Maki, Muraki, & Koyama, 2000). Together these results indicate that dopamine D1 receptors are not involved in pain perception in the central amygdala, but may affect this area in an attentional capacity (El-Ghundi, O'Dowd, & George, 2001).

D2 agonist quinpirole

In previous research it has been shown that D2 receptors have a role in nociception, because D2 antagonists enhance and D2 agonists reduce formalin-induced nociception (Fried et al., 2001). Conversely, systemic injections of both D2 agonists and antagonists appear to produce anti-nociception (Magnusson & Fischer, 2000). Consequently, effects of the D2 agonist, quinpirole on shock-enhanced startle were investigated.

In Experiment 21 quinpirole failed to have an effect during the shock sensitization paradigm as shock-enhanced startle, shock-reactivity or startle was not affected by the infusion of the drug into the central amygdala. Thus, it was unlikely that D2 receptors were involved in pain perception in this area. Furthermore, as shown in Experiment 27 the drug had no effect on fear expression to CS presentations. Although post CS presentation startle reactivity was enhanced, this was not statistically significant. The lack of a drug effect could not have been due to an ineffective dose or paradigm, since quinpirole significantly attenuated fear expression when infused into the basolateral amygdala. Together these results imply that D2 receptors do not affect pain or fear expression in the central amygdala. Dopaminergic neurons in the mesolimbic system originate in the ventral tegmental area and are important in the amygdaloid-based fear network. A significant link has been established between the VTA and the central amygdala (Weizman et al., 2003; Zarrindast, Nassiri-Rad, & Pazouki, 1999). This link is dopaminergic in nature and lesioning it significantly increased pain sensitivity (Gelowitz & Kokkinidis, 1999). However, the results of Experiments 21, 26 and 27 showed that dopaminergic neurotransmission does not appear to affect pain processing in the central amygdala, since neither D1 nor D2 agonists prevented foot shock-enhanced acoustic startle. Perhaps an investigation of the effects of D1 and D2 antagonists on pain

perception is warranted, as the infusion of the D2 antagonist eticlopride does not affect shock reactivity but does attenuate fear acquisition (Wood, 2006).

Summary of findings in the central amygdala

The main finding in this section was that glutamate neurotransmission in the central amygdala is important in the expression of fear arousal by footshock. A mechanism for this was a reduction in pain processing. This was evident in the suppression of fear during footshock but not during fear expression to a CS. NMDA appears to be the main glutamate receptor that is responsible for altering the perception of pain. The AMPA receptor seems to control pain and overall neural functioning during both paradigms. The GABAergic and dopaminergic systems or de novo protein synthesis did not appear to be involved in pain processing in the central amygdala.

How do the new findings fit in with the most recent knowledge about the amygdala circuitry?

The two most important findings from this thesis were that a GABA mechanism prevents fear expression to a US in the basolateral amygdala, and that a glutamate mechanism is responsible for fear expression in the central amygdala. They do so via different actions. It was proposed that a GABA mechanism is involved in fear arousal, while a glutamate mechanism prevents pain expression which in turn prevents increases in acoustic startle to footshock application. The final section of this thesis explored the neural pathways supporting these proposals.

The unconditioned stimulus, footshock, elicits pain, reflex and emotion (Borszcz, 1993, 1995; Borszcz & Leaton, 2003). During classical conditioning the emotive

component is paired to a 'non-aversive' stimulus (CS). This pairing occurs in the basolateral amygdala (Romanski, Clugnet, Bordi, & LeDoux, 1993) thereby indicating that information produced by the footshock must reach the basolateral amygdala (which must also receive information about the CS).

Fendt and Fanselow (1999) produced a clear visual representation of a proposed model. This model shows the connections between the amygdala and other areas in relation to US/ CS stimuli and the startle circuit (See Figure 8.1).

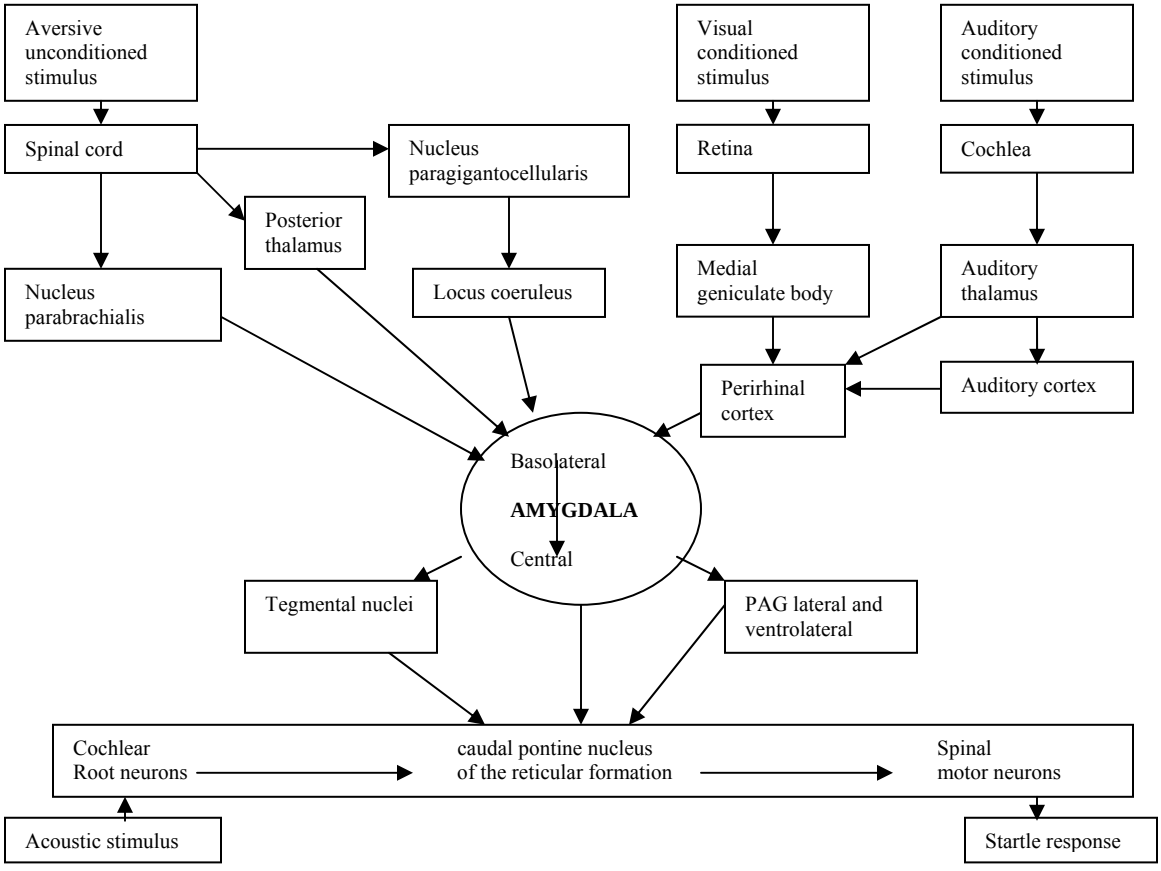

Figure 8.1: Neural connections proposed by Fendt and Fanselow (1999) demonstrating fear-potentiated startle neural substrates.

In this diagram (Figure 8.1), information from the aversive stimulus passes through the spinal cord to the nucleus parabrachialis and then on to the basolateral amygdala. The parabrachial nucleus has a significant function in transmitting nociceptive information from the spinal cord to the central amygdala. In Fendt and Fanselows' model, no connection between the parabrachialis and the central amygdala was evident. This implied that US information did not reach the central amygdala. Moreover, no information concerning visual or auditory stimuli reached the central amygdala meaning that US/CS associations could not be formed here. Evidence from lesion studies showed that the central amygdala was responsible for the expression of fear, and the basolateral amygdala for fear associations (Maren, Yap, & Goosens, 2001; Romanski, Clugnet, Bordi, & LeDoux, 1993). Fendt and Fanselow (1999) proposed a serial view of how unconditioned and conditioned stimuli could congregate at the basolateral amygdala, be processed there, and relayed to the central amygdala for expression (Jasmin, Burkey, Card, & Basbaum, 1997). This view did not support the earlier presented evidence that the central amygdala processes part of the unconditioned stimulus. The results of the shock sensitization experiments clearly show that the central amygdala processes part of the shock experience.

Other evidence also showed that the central amygdala is involved in fear processing. For example, inactivation of the central amygdala with muscimol prevented fear acquisition (Maren & Fanselow, 1996). Severing the connection between the lateral and central amygdala does not prevent synaptic plasticity arising from thalamic inputs towards the central amygdala (Wilensky, Schafe, & LeDoux, 2000) thereby indicating that the central amygdala can receive US/CS information without basolateral input.

Other researchers produced evidence that there are further pathways for processing the US (Samson & Pare, 2005). Thus, lesions of pathways carrying US information towards the basolateral amygdala did not prevent fear conditioning. This indicated that at least the central amygdala was potentially involved in processing US information. In particular, studies from the pain literature show clear evidence that the central amygdala mediates nociception (Manning, 1998; Manning, Martin, & Meng, 2003; Neugebauer, 2007; Neugebauer & Li, 2002; Shi & Davis, 1999). Studies investigating muscarinic acetylcholine receptors showed that the central amygdala coded the severity of the aversive stimuli (Manning, 1998; Neugebauer & Li, 2003). The central amygdala receives nociceptive information via the spino-trigemino-parabrachial-amygdaloid tract (Roozendaal et al., 1997). Lesioning the central amygdala reduces pain sensitivity (Jasmin, Burkey, Card, & Basbaum, 1997) prevents the shock sensitization effect (Balasubramanian, Pal, & Konar, 2006) and prevents fear acquisition (Hitchcock, Sananes, & Davis, 1989). Given these results, it is highly probable that the central amygdala does indeed process US inputs particularly the pain component of footshock.

It was proposed that the emotional component produced by footshock is processed in the basolateral amygdala. Earlier studies have clearly demonstrated that the basolateral amygdala receives US information via the parietal ventral area, rhinal cortex, the medial geniculate nucleus and also from other thalamic nuclei (Kim & Davis, 1993; Nader, Majidishad, Amorapanth, & LeDoux, 2001). Stimulation of the medial geniculate amygdaloid path produces long-term potentiation and supports auditory fear conditioning (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). Moreover, the indirect thalamo-cortico-amygdala connection also supports auditory fear conditioning (Clugnet &

LeDoux, 1990). For fear conditioning to occur, part of the US stimulus must become associated with a non-aversive stimulus. The lateral amygdala neurons are active during CS and US stimulation and it has been conclusively shown that the CS/US association is formed in this area (Bordi & LeDoux, 1992; Romanski, Clugnet, Bordi, & LeDoux, 1993). Once the association is formed most evidence shows that the actual consolidation of these associations is also processed in the basolateral amaygdala (Romanski, Clugnet, Bordi, & LeDoux, 1993). Moreover, the consolidated association is stored in the lateral amygdala (McGaugh, 2004). To demonstrate that it is the emotional component that has become associated with the neutral stimulus and this is not an expression of conditioning-related changes, Goosens, Hobin and Maren (2003) recorded neural firing patterns during various tests. They temporarily inactivated the central amygdala (which is necessary for the expression of fear behaviours (Fendt & Fanselow, 1999; LeDoux, Iwata, Cicchetti, & Reis, 1988; Maren, 2001)) to determine if it was the increased attention to the fear stimulus and fear expression itself that caused the neural firing in the lateral amygdala during fear expression. They still observed CS stimulated neural firing in the lateral amygdala, thereby disassociating the expression of fear behaviour from fear memory. These findings also indicate that the basolateral amygdala processes the emotional component of footshock.

Do these conclusions fit in with current views concerning the overall functioning of the amygdala? The answer is yes. According to the new model proposed by Pare, Quirk, and LeDoux (2004), both the central and basolateral amygdala do receive US/CS information. As was shown earlier, the older model did not account for the central amygdala being involved in neural plasticity, because it was proposed that US or CS

information was not received here. The central amygdala processed fear expression such as freezing and fear potentiated startle, and US/CS information flowed from the lateral amygdala through the central amygdala towards the brainstem. We now know that this area comprises several sub-nuclei (Pitkanen, Savander, & LeDoux, 1997), of which only the central-lateral receives information from the lateral amygdala. However, the central-lateral subnucleus does not project to the brainstem, but the central-medial subnucleus does. To complicate matters there is no direct connection between the central-lateral and central-medial. In the revised model this problem has been dealt with by proposing that information from the lateral amygdala reached the central-medial amygdala via intercalated cells. Moreover, CS/US information from the thalamus also reaches the central-medial area. The intercalated cells are sheets of thin layers of mainly GABAergic cells that inhibit/disinhibit information from the lateral towards the central-medial amygdala (Pare, Quirk, & LeDoux, 2004). Lesioning these, for example, affects the expression of fear extinction (Royer, Martina, & Pare, 1999).

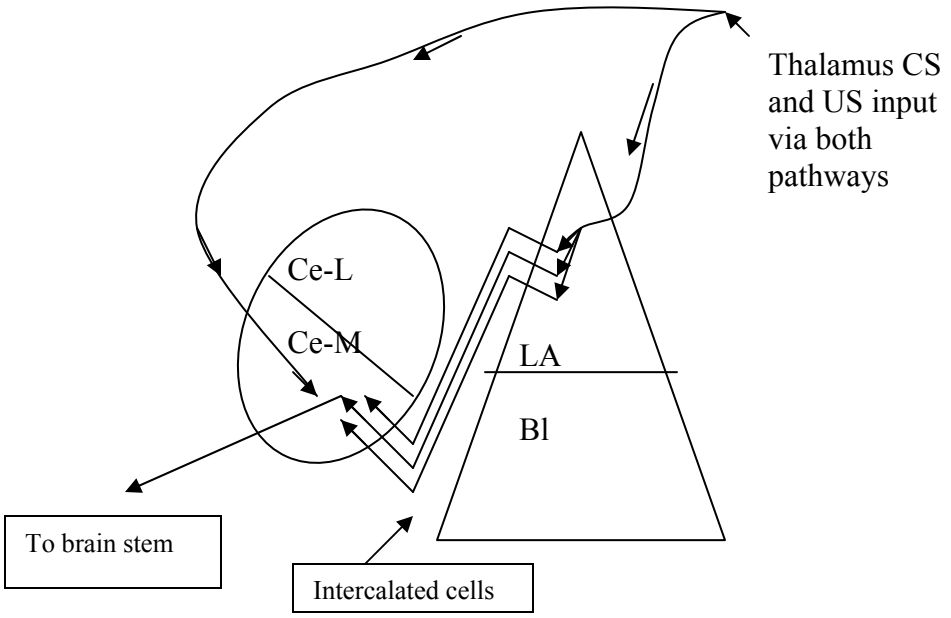

Figure 8.2: Model proposed by Paré, Quirk and LeDoux (2004) shows that CS/US information reaches both the lateral amygdala (LA) and the central medial amygdala (Ce-M), thus allowing plasticity in both areas.

This model allows for plasticity to occur in both the lateral and central amygdala, thereby supporting involvement of both the basolateral and central amygdala in the processing of footshock. Because both the basolateral and the central amygdala are involved in shock sensitization it appears that blocking the central amygdala via glutamate receptor inhibition is sufficient to prevent shock sensitization from occurring. Similarly, blocking of the basolateral amygdala via a GABA agonist is sufficient. These results provide evidence that both areas receive US information, but use different mechanisms to prevent the expression of the US.

By analysing the basolateral and central amygdala during footshock it has become clear that both areas contribute significantly to the perception of footshock. It has also

become clear that the combination of pain and fear are necessary for fear conditioning. Blocking pain in the central amygdala still allows the basolateral amygdala to form weak CS/US associations. This suggests that pain is important for fear conditioning but not necessary. Conversely, blocking fear in the basolateral amygdala prevents formation of all fear associations during conditioning thereby indicating that pain per se is not sufficient for fear conditioning to occur, conversely, fear is. Both the basolateral and central amygdala work in conjunction and perhaps in a parallel configuration with each other during fear conditioning.

Experimental limitations

Both paradigms have been well established in the literature (Davis, 1979, 1986, 1989, 2006; Fendt & Fanselow, 1999; Grillon & Davis, 1997). However while testing under drug influence some of the results were not clear. The results of Experiment 9 whereby dopamine agonists were infused into the basolateral amygdala during the shock sensization paradigm showed ambiguity. Similarly, the results reported in Experiment 24 whereby anisomycin was infused prior to conditioned fear expression showed ambiguity. Both experiments would benefit from repetition. It is also important to note that in Experiement 12 the number of rats used was relatively low (n=6).

Future research directions.

The significant effects of inhibition of glutamatergic neural transmission in the central amygdala warrant further investigation. Specifically, a dose response study for the AMPA antagonist CNQX during the shock sensitization and fear potentiated startle paradigm needs to be undertaken. This to investigate the minimum concentration required

to prevent shock sensitization and compare this to the minimum concentration required to prevent fear expression. Perhaps the same concentrations would also inhibit fear acquisition.

In the basolateral amygdala AP-5 blocks fear acquisition during US/CS1 (shock/tone) presentation and also during second-order CS2/CS1 (light/tone) presentation indicating that this is a long-term potentiation based learning deficit (Gewirtz & Davis, 1997). In the central amygdala, AP-5 reduces pain during footshock and also could prevent the formation of the association between the CS and US, perhaps because pain is not processed. For example, fear acquisition can be significantly reduced by AP-5 (Goosens & Maren, 2003). Conversley AP-5 should not block second-order fear conditioning, because no pain is involved in expressing the CS1 response, and thus AP-5 should not block the CS1-CS2 association.

Moreover, the NMDA effect should be further investigated via the use of the nitric oxide inhibitor L-NAME (N^{ω}-nitro-L-arginine methyl ester). This inhibitor prevents the production of nitric oxide which is released during NMDA receptor activation (Likhtik, Popa, Apergis-Schoute, Fidacaro Jr, & Pare, 2008). Discrete neural populations found in the amygdaloid area can produce nitric oxide (Bredt & Snyder, 1989). Like AP-5, L-NAME does not affect shock sensitization when infused into the basolateral amygdala but does prevent fear expression to a specific cue (Overeem, 2006). The infusion of L-NAME into the central amygdala should prevent the shock sensitization effect, but perhaps not fear expression to a specific cue. A direct relation between the inhibitory effects of L-NAME on NMDA receptors during noxious stimulation has been shown in spinal dorsal horn neurons (Budai, Wilcox, & Larson, 1995).

A final thought: when does fear become fear after footshock? Pain produced by footshock is conveyed by nociceptors in the footpads of a rat. These signals travel through various neural paths to the central amygdala. However, it is unlikely that fear is experienced in the footpads of the rat! Thus some remaining questions are when and where does the footshock signal become fear and how does it do this?

Summary

By using a simple shock sensitization paradigm various neural systems of negative affect and pain were investigated. The main findings were that, during immediate fear-arousal produced by footshock, GABA neurotransmission in the basolateral amygdala prevented the emotive value while glutamate neurotransmission in the central amygdala prevented pain. This suggests that both the central and basolateral amygdala process the unconditioned stimulus. These results also indicate that the shock sensitization paradigm is suited to investigate pain and negative affect in other areas. In 2004 Paré, Quirk and LeDoux suggested a new model for fear learning in which unconditioned stimulus information was processed in both the lateral and central amygdala. The results reported in this thesis support and extent this new model.

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